Handling of Inhaled Particulate Matter by Alveolar Macrophages in Children with Cystic Fibrosis

Thesis by

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In partial fulfilment of the requirements for the Degree of Doctor of Philosophy

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Statement of Originality

I, Norrice Mary Liu, confirm that the research included within this thesis is my own work, or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated. Previously published material is also acknowledged below. Photographs and images are generated originally from this research work, unless indicated in the text.

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Thesis Abstract

Background

Children with Cystic Fibrosis (CF) are vulnerable to the effects of inhaled carbonaceous pollutants, but the underlying mechanism remains unclear. In CF, bacterial phagocytosis by alveolar macrophages (AM) is impaired, with increased airway prostanoid levels. Since particulate matter (PM) clearance is mainly by AM phagocytosis, I hypothesised that in CF, the amount of inhaled carbon phagocytosed by AMs is reduced, with increased phagocytosis-inhibitory Prostaglandin-E2 (PGE_2) production, secondary to increased AM cyclooxygenase-2 (COX-2) expression.

Methods

Children with CF and healthy controls carried portable aethalometers to monitor their personal carbon exposure. AMs were obtained by sputum induction. Carbon areas of 50 AMs per participant were quantified using image analysis. Purified populations of AM were exposed to diesel exhaust particles (DEP) to compare phagocytosis in both groups. Addition of PGE_2 to the cell-DEP culture assessed its phagocytosis effects. AM COX-2 expression was determined by flow cytometry. Urinary prostanoid metabolites, as prostanoids production markers, were measured by mass spectrometry. Data were analysed by Mann-Whitney/Wilcoxon and paired t- tests.

Results

Despite no significant difference in median personal carbon exposure (CF 1394 ng/m^3, n=25 vs control 1587 ng/m^3, n=21; p=0.83); *in vivo* AM carbon was lower in CF (median 0.12 μm^2 vs 0.30 μm^2, $p<0.001$). AMs from both groups demonstrated considerable DEP uptake *in vitro* (CF median fold change 72 vs control 40); addition of PGE_2 attenuated DEP uptake (mean difference -3.88 μm^2, $p<0.001$). AM COX-2 expression was increased in CF (MFI median 10217 vs 8142; $p<0.05$), who also had higher urinary concentrations of PGE_2 metabolites (median 35965 pg/mg creat vs 15873 pg/mg creat, $p<0.001$).

Conclusion

AM phagocytosis of carbon is impaired in CF *in vivo*, but comparable with controls *in vitro*, suggesting inhibitory constituents are present in CF airways. Since COX-2 expression and PGE_2 production are increased in CF, I conclude that PGE_2 is inhibitory on CF AM function.

Contributions

Queen Mary University of London, London, UK:

Professor Jonathan Grigg wrote the grant proposal and secured funding for the study titled “Reducing the effects of air pollution in children with Cystic Fibrosis” from Barts Charity.

Dr Lisa Miyashita contributed to experimental designs, and assisted with sputum and placenta processing.

King’s College London, London, UK:

Dr Benjamin Barratt provided modelled data on air pollution exposure based on participants’ home addresses.

Jagellonian University Medical School, Krakow, Poland:

Professor Marek Sanak and his team analysed urine and sputum supernatant samples for eicosanoid metabolites.

IVL Swedish Environmental Institute, Sweden:

Dr Martin Ferm and Dr Marta Segura Roux analysed the nitrogen dioxide diffusion samplers.

Barts Health NHS Trust, London, UK:

Mr Graham McPhail performed electron microscopy for placenta samples.

University of Manchester, Manchester, UK:

Dr Carolyn Jones assisted in interpreting placental electron microscopy images.

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All other work and experiments in this study were undertaken by myself.

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Publications and Abstracts arising from work presented in this thesis

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The potential for air purification to reduce children's overall pollution exposure (ERS, Madrid, Sept 2019)

Reduced uptake of inhaled carbon in airway macrophages from children with cystic fibrosis (Researcher Links Workshops on Childhood Respiratory Disease in UK and China, Chongqing, Oct 2018)

Abstracts:

Liu N.M., Miyashita L., Grigg J. Why are children with Cystic Fibrosis (CF) vulnerable to air pollution? *William Harvey Research Day 2019 at Queen Mary University of London.*

Liu N.M., Miyashita L., Grigg J. The Protective Role of Airway Macrophages in Face of Air Pollution. *Am J Respir Crit Care Med* 2019;199:A6026

Liu N.M., Miyashita L., Grigg J. Reduced uptake of inhaled carbon in airway macrophages from children with cystic fibrosis *European Respiratory Journal* 2018 52: PA341

Liu N.M., Miyashita L., McPhail G., Thangaratinam S., Grigg J. Do inhaled carbonaceous particles translocate from the lung to the placenta? *European Respiratory Journal* 2018 52: PA3413

Liu N.M., Wan T., Russell-Jones E.C., Grigg J. Can masks protect you from air pollution? *Thorax* 72(Suppl 3):A162.2-A162

Liu N.M., Miyashita L., Russell-Jones E.C., Grigg J. Sputum purification revealing bacteria within airway macrophages of children with CF *Am J Respir Crit Care Med* 2018;197:A3670

Liu N.M., Miyashita L., Grigg J. Uptake of particulate matter by human placental macrophages. *VIB Macrophage Biology In The Single Cell Era* 2017

Miyashita L., Liu N.M., Mushtaq N., Grigg J. Exposure to particulate matter increases production of *Pseudomonas Aeruginosa* Virulence Factors *European Respiratory Journal* 2017 50: PA3321

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Glossary and Abbreviations

ABC	ATP-binding cassette
AM	Alveolar (airway) macrophage
AMBC	Airway macrophage black carbon
ASL	Airway surface liquid
ATS	American Thoracic Society
BAL	Broncho-alveolar lavage
BC	Black carbon
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CO	Carbon monoxide
COX-2	Cyclooxygenase-II
DC	Dendritic cell
DEP	Diesel exhaust particle
DMEM	Dulbecco's Modified Eagle Medium
DPBS	Dulbecco’s phosphate buffered saline
ERS	European Respiratory Society
EU	European Union
FBS	Foetal bovine serum
FEV1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
HAP	Household air pollution
IL	Interleukin
IQR	Inter-quartile range
LAQT	London Air Quality Toolkit
LPS	Lipopolysaccharides
MDP	Macrophage-dendritic progenitor
MHC	Major histocompatibility complex
NICE	National Institute for Health and Care Excellence
NO	Nitric oxide
NO2	Nitrogen dioxide
NOx	Nitrogen oxide
NSAID	Non-steroidal anti-inflammatory drug
O3	Ozone
PBS	Phosphate buffered saline
PG	Prostaglandin
PGD	Prostaglandin-D
PGE	Prostaglandin-E
PGJ	Prostaglandin-J
PM	Particulate matter
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute buffer
SO2	Sulphur dioxide
TLR	Toll-like receptor
TNF	tumour necrosis factor
TRAP	Traffic related air pollution
UK	United Kingdom
UV	Ultra-violet
VOCs	Volatile Organic Compounds
WHO	World Health Organisation

Chapter 1: Introduction

1. Introduction

1.1. Air pollution

Air pollution is a global public health burden, with half of the world's population residing in cities, megacities or peri-urban areas. According to World Health Organisation (WHO), 1.8 billion children and young people under the age of 15 across the world are breathing polluted air. WHO estimates that air pollution accounts for 600,000 paediatric deaths a year secondary to associated acute pneumonia. In Europe, more than 80% of city inhabitants are exposed to pollution levels exceeding the limits set by the WHO air quality guidelines [1]. In the United Kingdom (UK), numerous cities regularly breach the European Union (EU) legal limits and WHO guidelines for air pollutants (Table 1.1) [2].

Pollutants	WHO guidelines (averaging period)	EU legal limits (averaging period)	Main sources
Particulate matter (PM_{10})	50 µg/m^3 (24 hours) 20 µg/m^3 (1 year)	50 µg/m^3 (24 hours) 40 µg/m^3 (1 year)	Transport (exhaust, tyre, brake wear), combustion, industrial processes and construction
Particulate matter ($PM_{2.5}$)	25 µg/m^3 (24 hours) 10 µg/m^3 (1 year)	25 µg/m^3 (1 year)	
Nitrogen Dioxide (NO_2)	200 µg/m^3 (1 hour) 40 µg/m^3 (1 year)	200 µg/m^3 (1 hour) 40 µg/m^3 (1 year)	Transport, combustion
Ozone (O_3)	100 µg/m^3 (8 hours)	120 µg/m^3 (8 hours)	Reaction of hydrocarbons, nitrogen oxides and volatile organic compounds in sunlight
Sulphur dioxide (SO_2)	500 µg/m^3 (10 min) 20 µg/m^3 (24 hours)	350 µg/m^3 (1 hour) 125 µg/m^3 (24 hours)	Coal combustion and road transport

Table 1.1. WHO guidelines, EU limits and main sources of ambient (outdoor) air pollutants. Adapted from *World Health Organization (WHO) ambient (outdoor) air quality and health fact sheet* (updated Sept 2016), *European Commission Air Quality Standards* (updated Sept 2017), and *Lethal & Illegal, Solving London's Air Pollution Crisis* by Institute for Public Policy Research, November 2016.

It is worth noting that national and international guidelines employ different risk assessment tools, resulting in different pollutant limits; risk assessment is based on the target population's social standards, background air pollution severity, and the extent and feasibility of risk adversity.

In the UK, 40,000 excess deaths per year are attributable to air pollution – these comprise new disease incidence secondary to chronic air pollution exposure, and exacerbations of established diseases; costing the nation over £20 billion in health and social services each year [3]. There is robust epidemiological evidence that air pollution impacts on every stage of life, and is linked to adverse effects on the respiratory, cardiovascular, and neurological systems [3-5]. Children and young people are more vulnerable because air pollutants can impede the normal development of their organs, particularly the lung – rendering children with respiratory diseases more susceptible. Moreover, children's immature immune and metabolic systems are also less capable of tackling inhaled pollutants.

1.1.1. Major air pollutants

1.1.1.1. Particulate matter

Particulate matter (PM) is made of solid and liquid organic and inorganic particles suspended in air. PM is categorised into PM_{10} and $PM_{2.5}$ based on its aerodynamic diameter – less than 10 or 2.5 micrometres (μm) respectively. For reference purpose, the diameter of human hair ranges from 17 to 181 μm. Coarse particles settle mainly in the upper airway, whereas finer particles are regarded as more harmful because they can reach the lower smaller airways and alveoli, and have

the potential to penetrate the systemic circulation and translocate to distant organs. The composition and size of PM are determined by its source. A key constituent of PM is black carbon (BC), which forms the core of these particles, and is responsible for the black appearance of “soot”. Transition metals and aromatic hydrocarbons are amongst other components of PM [6].

“Primary” PM is defined as fine particles that are emitted directly to the atmosphere, including anthropogenic sources such as traffic-related air pollution (TRAP), coal and oil combustion in power stations and factories, biomass burning, construction work and agriculture. “Secondary” PM is formed by photochemical reactions of pollutants, such as nitrogen oxides (NO_X), in the atmosphere.

1.1.1.2. Nitrogen oxides

Nitrogen oxides (NO_X, including nitric oxide, NO and nitrogen dioxide, NO_2) formation can occur in natural or anthropogenic ways, such as when nitrogen reacts with oxygen during lightning, microbial processes, and fossil fuel combustion; the last being the biggest contributor to urban NO_X, responsible for 40% of Europe’s total emission [7].

1.1.1.3. Ozone

Ozone (O_3) is generated from photochemical reactions between NO_X, volatile organic compounds, and oxygen under strong ultraviolent (UV) light. Its levels therefore peak on hot, sunny, windless days. O_3 is able to travel far and accumulate in the atmosphere distant from its source.

1.1.1.4. Sulphur dioxide

Sulphur dioxide (SO_2) is a colourless acidic gas formed during volcanic activity, and also as a by-product when sulphur-containing fuels, such as coal and heavy oils, are burned. The largest contributor of SO_2 in the developed world is power generation.

1.1.2. Outdoor (ambient) air pollution

The major sources of ambient pollutants are gasoline- and diesel-powered engines (producing PM, NO_X), vehicle tyre and brake wear (producing PM), power stations and factories where coal combustion and biomass burning (producing PM, NO_X, SO_2) occur [8-10].

Globally, 93% of children are exposed to $PM_{2.5}$ levels higher than WHO air quality guidelines [11]. Road traffic has been increasing in urban areas over the last few decades, with active forms of transport such as walking and cycling being on a decline [3]. In the UK, 50% of NO_2 emissions are generated on the roads [12], with about half the cars and the majority of heavy vehicles and trains being powered by diesel engines [13], which generate the most PM and NO_X amongst all engine types. Diesel soot is categorised as carcinogenic by WHO [14]. Children are inevitably exposed to outdoor pollution during their commute to and from school and outdoor activities. Many nurseries and schools in the UK are located close to highly polluted roads where NO_X concentrations regularly breach the legal limits (40 $\mu g/m^3$ annual mean or 200 $\mu g/m^3$ 1-hour mean) [12,15].

1.1.3. Indoor air pollution

Indoor air pollution is mainly caused by inefficient fuel or biomass combustion during cooking and heating, generating NO_2 and carbonaceous PM; it can also come from ingress of outdoor pollutants. Other common indoor air pollutants include carbon monoxide (CO), SO_2 and volatile organic compounds (VOCs). These are found in cigarettes, building materials, furnishing chemicals, insecticides, cleaning products, dust, and mould. Across the globe, WHO reports that household air pollution (HAP) increases the risk for paediatric pneumonia twofold, and is liable for 45% of pneumonia deaths in children aged less than 5 years. In high income countries, the urban population tend to dwell indoors for the majority of time, making indoor air pollution, even at low levels, a significant factor in health effects.

Summary: Indoor and outdoor air pollution

- Air pollution is a growing global public health problem, mainly caused by incomplete fossil fuel or biomass combustion.
- Traffic-related air pollution (TRAP) is one of the biggest sources of pollutants such as particulate matter, nitrogen oxides, ozone and sulphur dioxide. For example, in the UK, TRAP is responsible for 50% of nitrogen dioxide emissions.
- There are national and international guidelines on air pollutant limits – urban cities such as London are regularly non-compliant with such limits.
- There is strong epidemiological evidence linking air pollution exposure to negative health effects, affecting every stage of the life course. In the UK, 40,000 excess deaths are attributable to air pollution each year.

1.2. The body's defence mechanism against air pollution

1.2.1. Barrier mechanisms: airway epithelium and surface liquid layer

In a healthy lung, the luminal respiratory tract is lined by epithelial cells, which form the body's first line of defence – this airway epithelium acts as a physical and chemical barrier. Its surface is lined with the airway surface liquid (ASL) which is important for airway homeostasis. ASL is responsible for regulating ciliary function, mucociliary clearance, and anti-pathogenic activities. The ASL is made of two layers – the mucus layer is dominated by gel-forming mucins, which trap inhaled particles for mucociliary clearance; the periciliary liquid layer maintains an optimal distance between the mucus and the underlying epithelia – this layer contains mucins bound to the cilia, forming a tight mesh which prevents the invasion of inhaled particles [16,17]. The two major types of epithelial cells are goblet (mucin producing) and columnar (ciliated). Goblet cells produce the mucin that lines the epithelium, while ciliated cells propel the particle-containing mucus and secretions upwards towards the pharynx (figure 1.1).

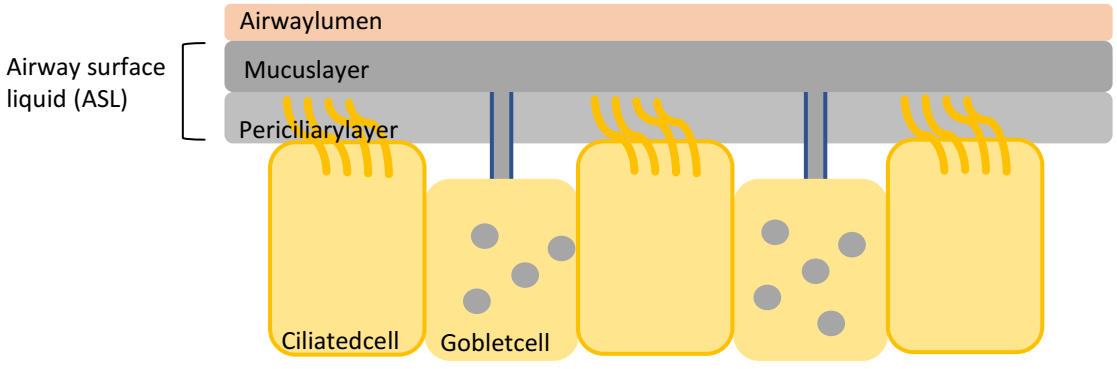

Figure 1.1. Airway epithelium and airway surface liquid layer – a physical and chemical first line of defence of the respiratory tract.

1.2.1.1. Inflammatory mechanisms

Other than a physical and chemical barrier, the epithelium also contributes to host defence by releasing arachidonic acid derivatives [18] and producing inflammatory mediators – leading to differentiation, activation and chemotaxis of inflammatory cells. Epithelial cells can produce various cytokines (interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 11 (IL-11), and tumour necrosis factor α (TNF-α)) when exposed to stimulants such as diesel exhaust particles (DEP) and NO_2 [19] [20]. Not only can air pollutants cause cytokines release and pro-inflammatory cell signalling, they can also damage epithelial cells. DEP and NO_2 can reduce ciliary beat frequency [21] [20] and damage the cell membrane, thereby increasing cell permeability [19]. Traditionally, it was thought that the fine physical diameter of DEP (PM) was the main reason for their negative health effects. However, these effects are seen in filtered liquid forms of DEP as well, suggesting that their chemical composition is just as significant [19]. Furthermore, exposure to engine exhausts can lead to increased peripheral leucocytes, neutrophils and airway eosinophils [22], all contributing to the inflammatory cascade.

1.2.2. Cellular removal mechanisms

1.2.2.1. Dendritic cells

Dendritic cells (DC) are antigen-presenting cells. A tight network of DCs is present within the airway epithelium, most of these reside in an immature state [23]. By extending their dendrites between epithelial cells, they sense and trap invading PM, followed by transporting them to the lymphatic system and stimulating T-cells to secrete cytokines, acting as a bridge between the innate and adaptive immune

systems. Indeed, it has been shown that DEP can increase the production and maturation of DCs, and recruitment of neutrophils and monocytes [23].

1.2.2.2. Alveolar space

Particulate matter which escapes the aforementioned mechanisms will be confronted by the phagocyte system. It is long recognised that monocytes, macrophages and dendritic cells are closely related. In mice, DC and monocytes arise from a common precursor – the macrophage-dendritic progenitor (MDP) [24]. In humans, the differentiation is less well understood. The role of monocytes is to sense the environment, and to govern and maintain the populations of macrophages and DCs. They can alter their phenotype according to the environmental need – for example, upon infection, monocytes can differentiate into macrophages and DCs. Different subsets of monocytes are distinguished by their surface markers, and they play different roles. Circulating anti-inflammatory monocytes maintain a stable composition of monocytes – during steady state, they become tissue resident macrophages; but they can differentiate into anti-inflammatory macrophages for tissue damage repair upon inflammation [25]. On the other hand, inflammatory monocytes recognise pathogens and apoptotic debris, phagocytose, and produce cytokines to initiate inflammation; they can also differentiate into inflammatory macrophages to further help remove pathogens and debris [25]. Classical monocytes seem to be the main source of monocyte-derived DCs, but all subsets are able to differentiate to macrophages [26].

1.2.2.2.1. Alveolar macrophages

Macrophages are the major effector cells in immune responses. Lung macrophages are categorised into alveolar macrophages (found on alveolar epithelial surface), and interstitial macrophages (found within alveolar walls and lung parenchyma) [27,28]. A subgroup of lung macrophages are found on the airway epithelium – airway macrophages – these have similar phenotypical and functional characteristics to alveolar macrophages, most likely because they are in fact alveolar macrophages migrating up the bronchial tree [27].

Alveolar macrophages (AM) are the resident phagocytes in the lower airways. AMs can adapt and coordinate inflammatory responses depending on the host requirements – initiating either an inflammatory response when invaded by pathogens and particles, or an anti-inflammatory response when clearing and degrading apoptotic debris [29]. These opposing pro- and anti-inflammatory responses suggest that macrophage function is regulated sophisticatedly, dictated by the environment. AMs are divided into classic- (M1) or alternatively- (M2) activated phenotypes [30]. M1-activated macrophages are pro-inflammatory, they are polarised following lipopolysaccharides (LPS) stimulation; whereas M2-activated macrophages are inflammation-resolving, and are polarised after cytokine stimulation [31] (figure 1.2).

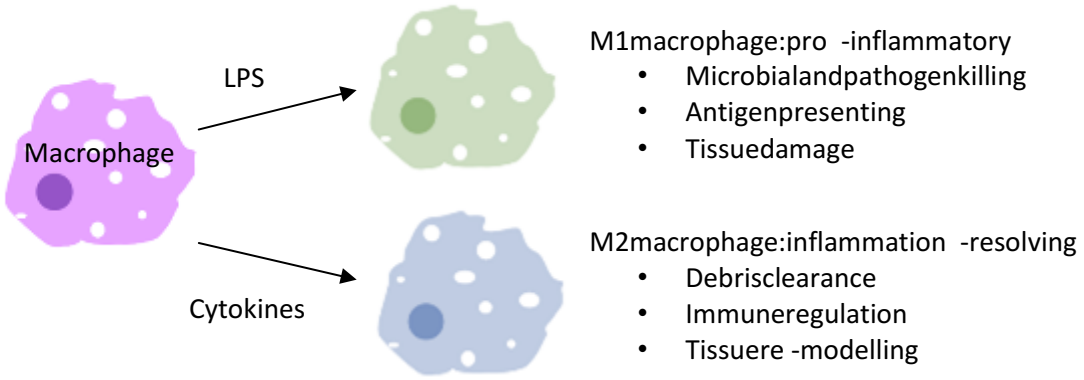

Figure 1.2. Activation of macrophages into M1 (pro-inflammatory) and M2 (inflammation-resolving) phenotypes depending on the environmental stimuli.

Despite the traditional view of AMs being monocyte-derived terminally differentiated cells which are replaced by circulating monocytes following acute lung insult [32], newer studies have suggested that they can self-renew and proliferate locally [33]. Not only can conditions such as pulmonary fibrosis and allergic inflammation promote monocyte-derived macrophage infiltration, they can also enhance mitogenic signalling and macrophage proliferation, leading to an increased number of macrophages [33]. Self-renewed macrophages have been shown to be protective against inflammation, while monocyte-derived macrophages can aggravate it [33].

AMs recognise insults such as damaged cells, apoptotic cells, pollution particles and microbes. They sample their environment through opsonic (FcR, CR3) and non-opsonic receptors (lectin, scavenger, and toll-like receptors) [34] (figure 1.3).

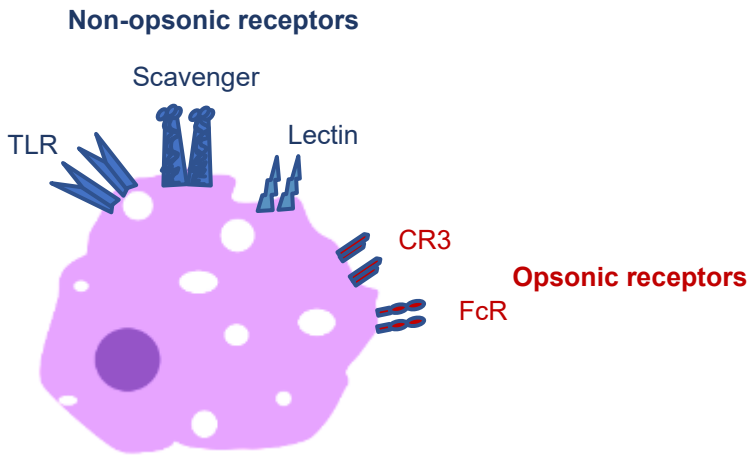

Figure 1.3. Macrophage receptors: non-opsonic receptors (in blue) recognise targets directly; opsonic receptors (in red) require opsonin to coat the target, allowing for recognition indirectly.

Opsonic recognition is indirect, requiring opsonin to coat the substance, thereby allowing for its recognition by receptors; non-opsonic recognition is when receptors directly recognise the substance. For example, toll-like receptors (TLRs) recognise gram-positive and -negative bacteria and particulate matter, whereas scavenger receptors (e.g. macrophage receptor with collagenous structure, MARCO) recognise lipoproteins and particulate matter, and lectin receptors recognise components of fungi [34]. AMs internalise material through receptor-mediated endocytosis and extension of pseudopodia. The pseudopodia surround the material, forming a phagosome which fuses with lysosomes to become a phagolysosome [35] (figure 1.4).

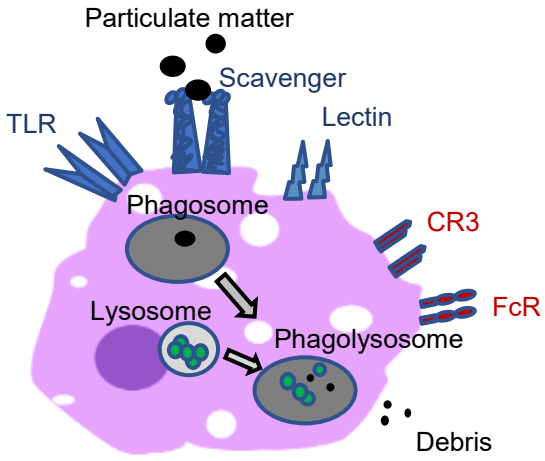

Figure 1.4. An alveolar macrophage encountering inhaled particulate matter (or any other foreign insults) via surface scavenger receptors, followed by the formation of a phagosome, fused with lysosomes to form a phagolysosome before elimination of the invading substance.

Autophagy is one of the mechanisms through which degradation of engulfed cytoplasmic contents occur: double-membraned vesicles enclose intracellular components for lysosomal degradation. Autophagy is also associated with antigenic fragment delivery to major histocompatibility complex (MHC) class II molecules, which are expressed on the macrophage surface, for presentation to T-cells [36,37]. It has been reported that increased autophagy following exposure to PM may protect against PM-induced cytotoxicity [38,39].

Additionally, AMs can induce inflammation and recruit neutrophils, which when activated, will initiate monocyte recruitment and contribute to insult elimination [34]. Alternatively, macrophages can eradicate phagocytosed material through programmed cell death [40].

AMs patrol the surface of the lower airways, neutralising and phagocytosing unwanted substances such as inhaled carbonaceous particulate matter, thus preventing their accumulation within the lungs (figure 1.5). Inhaled PM is phagocytosed in a dose-dependent manner [41,42]. As mentioned in section 1.1.1.1., coarser PM tends to deposit in the larger proximal airway, while ultrafine PM can penetrate further into smaller distal airways and alveolar space. Therefore, normal AM function is crucial in preventing PM accumulation within the lungs, limiting PM exposure to other airway cells and reducing its negative effects on lung health.

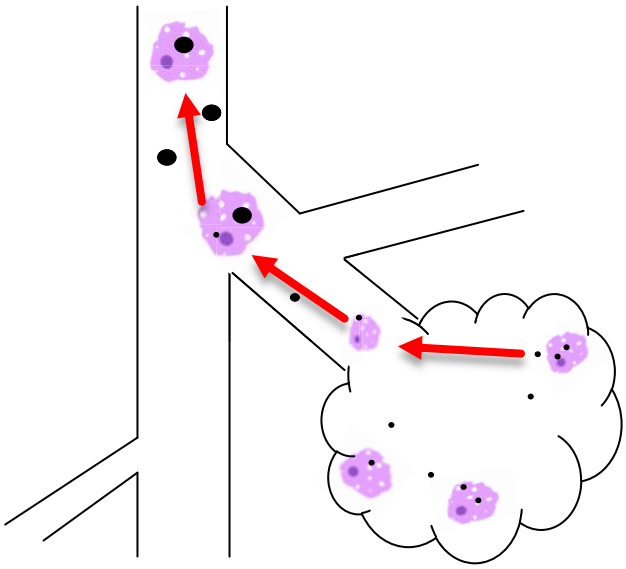

Figure 1.5. Alveolar macrophages patrolling the airway, phagocytosing unwanted substances along the way.

AM phagocytosis of inhaled PM is further discussed in section 1.5.3.

Summary: The body's defence mechanism against air pollution

- The respiratory tract is the first point of contact of inhaled particulate matter.
- The epithelium and airway surface liquid form the first line of defence, acting as a physical-chemical barrier which traps particulate matter.
- Particulate matter that overcome the physical-chemical barrier will be confronted by cellular removal in the lower airways.
    - Alveolar macrophages are the main phagocytes for inhaled particle removal, facilitated by cell surface receptor recognition of particle, followed by phagolysosomal formation and particle destruction.
- The epithelium and alveolar macrophages both contribute to the inflammatory processes (e.g. cytokine release, neutrophils recruitment) associated with exposure to inhaled particulate matter.

What is still unknown?

- The consequences of residual particulate matter in the distal airways, not phagocytosed due to impaired AM function or saturation of AM functional capacity, remain unclear.

1.3. Health effects of air pollution across the life course

There is robust epidemiological evidence showing that the adverse effects of air pollution transpire across the human life course [3]. While some effects may be reversible, others have long-term impacts. However, to determine the health effects of each component of air pollution is challenging because many pollutants share common origins – with the most common source being fossil fuel combustion and vehicle engines.

1.3.1. Antenatal effects and mechanisms

Fetal cells are fast replicating and sensitive to external insults such as air pollution. Organogenesis of the lung begins at 4 weeks of gestation, with alveolar development starting at around 36 weeks, continuing into early adulthood. Although it is challenging to separate the effects of maternal air pollution exposure from those of post-natal exposure, independent associations of antenatal air pollution exposure and impaired lung development and function have been reported [43]. Intrauterine exposure to NO_2, particularly during the second trimester, is associated with reduced forced expiratory volume in 1 second (FEV_1) later in childhood – Morales *et al.* [44] reported that an interquartile range (IQR) increase in NO_2 exposure would result in -28.0 mL of FEV_1, with a relative risk of having low lung function, defined as < 80% of predicted FEV_1, of 1.30. Other studies have described a link between maternal exposure to traffic-related air pollutants and adverse birth outcomes such as increased infant mortality, premature birth, restricted fetal growth and low birth weight at term [45,46]. Worryingly, the increased risk of low birth weight could occur at $PM_{2.5}$ levels below the EU limit recommendation of 25 $\mu g/m^3$ per year [46,47].

There are several potential mechanisms through which fetal development is affected. It is generally recognised that adverse fetal outcomes are related to underlying placental insufficiency. Fetal health is closely related to maternal health – oxidative stress and inflammation within maternal airways could result in haemodynamic and vascular changes, leading to reduced placental circulation and fetal nutritional delivery [45]. Indeed, there is evidence that PM exposure has a dose-dependent effect on the development of pre-eclampsia, a condition in which the placenta plays an important role [48]. Another probable mechanism is particle translocation from the maternal lung, via the circulation, to the placenta and fetus, where particle toxicity is exerted directly [45]. It is known that PM is cytotoxic to human placental cells, affecting their gene expression profile and cell signaling [49]; placental epigenetic modification and fetal re-programming can result in predisposition to diseases in adulthood [50]. For example, it has been shown that antenatal exposure to $PM_{2.5}$ is associated with increased risk of asthma later in life [51]. Further discussion on particle translocation outside the respiratory system can be found in section 1.4.4.

1.3.2. Postnatal effects

Air pollution can alter children's developmental trajectory as the human body continues to grow until the end of adolescence or early adulthood. The first 6 years of life represent a crucial time for lung development – during which formation of over 80% of alveoli occurs. The negative associations between TRAP and children's lung growth, lung function, and respiratory health are well recognised.

Antenatal exposure to air pollution is associated with increased respiratory need and airway inflammation in newborn infants [52], leaving them more susceptible to wheeze and bronchiolitis, which are worsened by postnatal outdoor pollutant exposure [53-55]. The combination of an infant's physical proximity to traffic exhaust fumes during outdoor travel (due to the lower level of their prams) and their higher relative minute ventilation compared to adults, render them more vulnerable to pollution's adverse effects.

1.3.2.1. Lung development and function

Residing in areas with high concentrations of PM and NO_2 can lead to suppression of lung function growth in school children [56,57]. Those with existing chronic conditions, or living in close proximity to road traffic are most susceptible [54,58]. A study in southern California showed that children and young people residing within 500 m of a motorway had significant growth deficit in FEV_1 (-81 mL over 8 years), compared to those living more than 1500 m away [59] (figure 1.6).

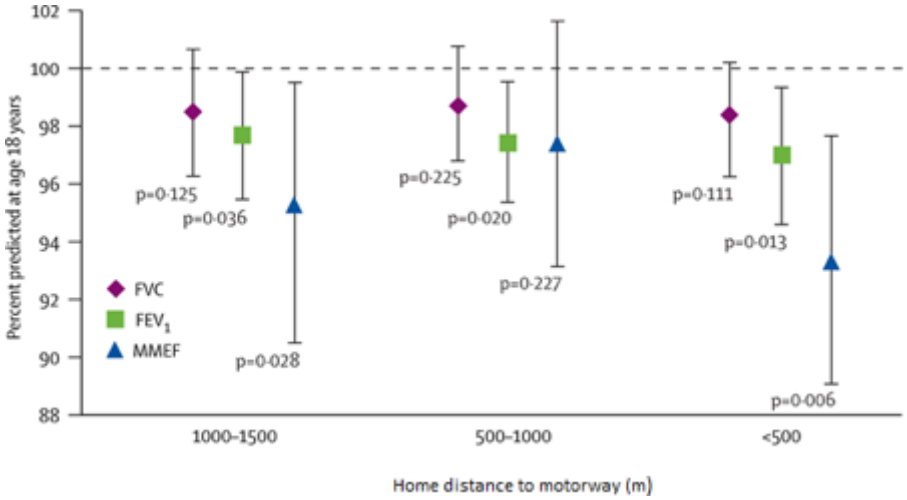

Figure 1.6. Percent-predicted lung function at 18 years of age, comparing different distances between home and a motorway – data from The Children's Health Study in southern California, showing significant Forced Expiratory Volume in 1 second (FEV_1) deficit in those living within 500 m of motorway; with insignificant effects on forced vital capacity (FVC) at all distances, and maximal mid-expiratory flow (MMEF) at all distances except for <500 m. Reproduced with permission from Gauderman *et al* [59], the Lancet.

Urman *et al.* [60] reported that increasing NO_X by 17.9 ppb led to -1.56% in forced vital capacity (FVC) and -1.1% in FEV_1 in children's lung function. Encouragingly, improvement in air quality can reverse or halt this reduction in lung growth and function. The Children's Health Study in southern California [61] demonstrated that the mean growth of FEV_1 over a 4-year period improved by 91.4 mL per 14.1 ppb reduction in NO_2, 65.5 mL per 8.7 µg/m^3 reduction in PM_{10}, and 65.5 mL per 12.6 µg/m^3 reduction in $PM_{2.5}$; with comparable changes seen in FVC (figure 1.7).

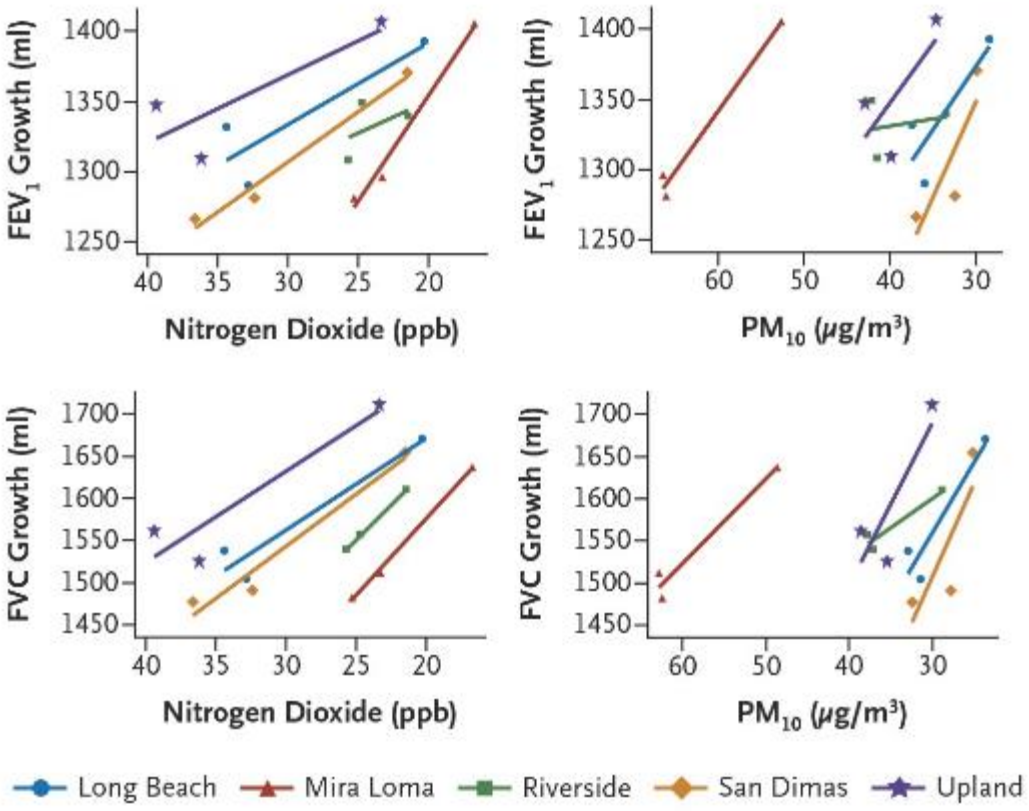

Figure 1.7. Mean FEV_1 and FVC growth in children over 4 years, plotted against levels of nitrogen dioxide and PM_{10} for different communities in southern California. Data from the Children's Health Study, showing improvement of both FEV_1 and FVC growth with reduction of nitrogen dioxide and PM_{10}. Adapted from and reproduced with permission from Gauderman *et al* [61], Copyright Massachusetts Medical Society.

1.3.2.2. Respiratory symptoms in previously healthy children

Evidence linking air pollution to asthma is robust – not only is air pollution strongly associated with exacerbations of pre-existing asthma, it can also result in new-onset asthma [6]. At high concentrations, NO_2 and $PM_{2.5}$ can cause airway inflammation and hyper-responsiveness [62], and may lead to oxidative stress, inflammation and remodelling in the airways, thereby predisposing children to sensitisation [6], bronchitis and wheeze [56]. A meta-analysis concluded that NO_2 exposure is linked to new onset asthma, while $PM_{2.5}$ exposure is connected to new onset wheeze [58], with its short-term variations closely associated with asthma exacerbations [63]. In addition to asthma, short term exposure to TRAP can aggravate respiratory infections and result in more frequent emergency department attendances in young children [64]. Reassuringly, as with lung function improvement, ambient NO_2 and PM reduction can decrease incidence of asthma, bronchitis, respiratory infections and hospital admissions [65,66].

1.3.2.3. Effects of air pollution on other body systems

There is evidence to support a link between air pollution and neurological development and function in children, resulting in reduced neurocognitive ability [67-69], due to disruption of brain maturation by high levels of TRAP [70]. These cognitive effects can linger into adulthood, with dementia and Parkinson's disease also associated with air pollution [71,72].

As mentioned above, air pollution can affect fetal wellbeing and result in low birth weight, which has an inverse association with cardiovascular morbidity and mortality in adulthood [73]; with evidence of increased pulmonary arterial pressure and diastolic

blood pressure following chronic air pollution exposure [74,75]. PM is also associated with DNA damage and genomic alterations, thus contributing to the development of lung carcinoma in non-smokers [22].

With regards to the endocrine system, animal studies show strong associations between air pollution exposure and type 2 diabetes, with epidemiological studies supporting this link in humans [76]. Indeed, Thiering *et al.* [77] demonstrated that NO_2 and PM exposure was related to insulin resistance in healthy children.

Summary: Health effects of air pollution across the life course

- Antenatal exposure to air pollution can affect the unborn fetus, resulting in premature delivery, low birth weight at term, and infant mortality.
- Postnatal exposure to air pollution is associated with reduced lung growth, lower lung function, increased risk or respiratory infections, pre-school wheeze, new onset asthma, and asthma exacerbations.
- There is evolving evidence suggesting air pollution also impacts on children's neurocognitive development.
- Exposure to air pollution is associated with cardiovascular and endocrine co-morbidities all through to adulthood.

What is still unknown?

- The specific impact of air pollution exposure on children with respiratory conditions (e.g. cystic fibrosis) remains unclear.
- Whether the reported remote effects of air pollution on extra-pulmonary organs are secondary to direct or indirect (or both) actions remain unclear.

1.4. Mechanisms underlying air pollution's negative health effects

1.4.1. Oxidative stress

Air pollutants such as $PM_{2.5}$ and NO_X are potent oxidants and can generate reactive oxygen species (ROS). ROS are usually formed as by-products of normal oxygen metabolism, their levels are controlled by the body's production of antioxidants and detoxification enzymes which counteract ROS production. Pollutants and ROS interact and undergo radical and redox reaction cycles in the epithelial lining fluid (ELF) [78]. When there is high concentration of ROS, the protective antioxidant response becomes inadequate – the imbalance between ROS generation and antioxidants is known as oxidative stress. High level of ROS can cause biological processes (e.g. inflammation) with various cytotoxic effects, leading to cell damage and cell death [79]. Not only can oxidative stress occur in individuals inhaling pollutants, maternal oxidative stress during pregnancy can also result in haemodynamic changes, affecting placental circulation [45], thus influencing fetal wellbeing (as discussed in section 1.3.1.). Evidence of oxidative stress related to air pollution can also be seen in extra-pulmonary organs such as the heart [80], and one mechanism by which inhaled air pollutants can affect distant organs.

1.4.2. Inflammatory responses

Further to section 1.2.1.1., air pollutants, particularly those from TRAP, are known to promote inflammatory cytokines production [81,82]. In particular, interleukin 1-beta (IL-1β), interleukin 6 (IL-6), and tumour-necrosis-factor alpha (TNF-α) productions are intensified by PM exposure [83]. The inflammatory response, and any subsequent

respiratory symptoms, vary according to the chemical composition and source of PM [84], with the mix of diesel exhaust particles (DEP) from TRAP being amongst the most toxic [85]. Indeed, DEP has been shown to stimulate IL-6 and IL-8 expression in nasal cells, thereby causing and/or aggravating conditions such as allergic rhinitis and rhinosinusitis [86].

1.4.3. Structural effects on epithelial cells

By exposing epithelial cells (A549 adenocarcinomic human alveolar basal epithelial cell line) to ultrafine titanium dioxide (TiO_2) particles, Stearns *et al.* [87] demonstrated that the particles were able to adhere to the cells despite multiple *in vitro* washing; the epithelial cells could also internalise TiO_2 particles by means of endocytosis, where they aggregated mostly in membrane-bound vacuoles. Other research also supports that endocytosis is a crucial process in the uptake of nanoparticles by human alveolar epithelial cells [88]. Not only can nanoparticles induce lung oxidative stress and inflammation, they can also cause biomechanical changes in human alveolar epithelial cells – leading to reduced actin stress fibres, reduced cell stiffness and traction forces; resulting in altered cell permeability [89].

1.4.4. Translocation of pollution particles across the air/tissue interface

As postulated in section 1.3.1., despite inhaled air pollutants largely ending up in the respiratory tract where they are phagocytosed and removed, they can also deposit in the airway, and may migrate elsewhere in the body. Non-phagocytosed PM may leach into the systemic circulation, causing oxidative stress and inflammation at distant organs (figure 1.8).

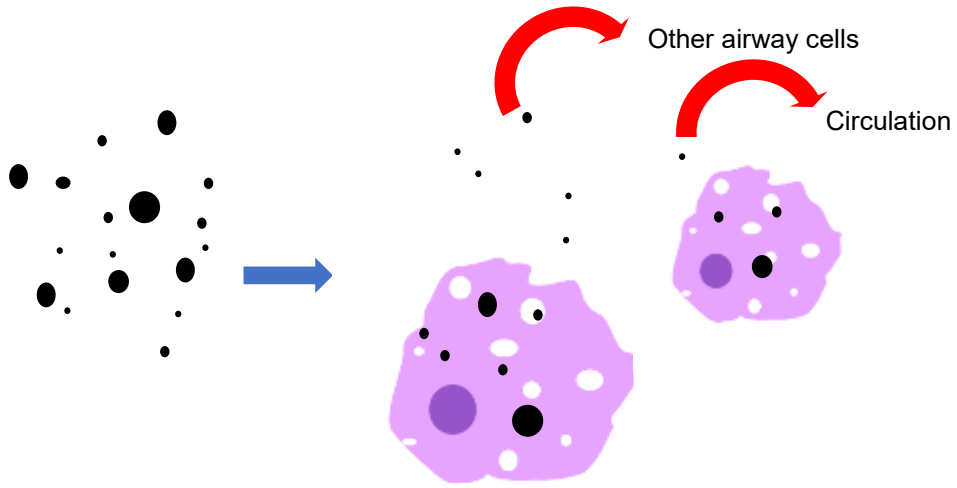

Figure 1.8. Inhaled particles encountering alveolar macrophages in the distal airways. Non-phagocytosed particulate matter will either stay within the airway and invade other airway cells, or leech into the systemic circulation, resulting in oxidative stress and inflammation at a distant site.

Using animal models, Nemmar *et al.* [90] showed that ultrafine particles were able to penetrate tissue compartments, travel through the circulation, and be taken up by mouse erythrocytes; Oberdorster *et al.* [91] showed that particles could deposit along the respiratory tract lining in rats, followed by translocation to extra-pulmonary organs within 24 hours of inhalation exposure; Campagnolo *et al.* [92] reported increased production of placental inflammatory cytokines following translocation of inhaled nanoparticles to placenta in mice. For human studies, *in vitro* work has demonstrated invasion and accumulation of nanoparticles in placental tissue [93], with the smaller particles (3-4 nm) being able to penetrate deeper [94]; while a human *ex vivo* study showed that nanoparticles with a diameter up to 240 nm were able to cross the placental barrier and be taken up by placental tissues [95]. Particle translocation is another possible explanation for pollutants' adverse effects on the developing fetus.

Besides translocating via the circulation, ultrafine particles instilled intra-nasally can travel along the olfactory nerve into the central nervous system, by-passing the blood-brain barrier [96]. Indeed, a recent study showed that magnetite pollutant particles with diameter <200 nm, arose from inorganic high temperature combustion, could enter the brain via the olfactory bulb [97].

To this date, however, the transport of particles beyond the human respiratory system remains unclear.

Summary: How does air pollution affect health?

- Inhaled air pollutants such as particulate matter and nitrogen oxides are potent oxidants and can cause oxidative stress, resulting in cell damage and/or death.
- Air pollutants promote airway inflammation by enhancing the production of inflammatory mediators.
- Inhaled particles that are not phagocytosed by alveolar macrophages can cause local inflammation and damage in other airway cells, but may also translocate via the circulation and exert their toxic effects directly at a distant site – as demonstrated by animal models.

What is still unknown?

- The direct and local consequences of non-phagocytosed inhaled particles within the human airway remain unclear.
- There is a lack of data on the feasibility, mechanism, and consequences of particle translocation in humans.

1.5. Measuring air pollution exposure in children

As discussed so far, air pollution poses short-term and long-term health effects across the life course, with children, particularly those with respiratory conditions, being most vulnerable. In order to further understand the impact of air pollution on the population, it is important to measure their pollution exposure.

Measurement and quantification of an individual's exposure to air pollution can be challenging. Long-term exposure depends on numerous variables such as weather, individual's daily routine and behaviour (e.g. nature of work, mode of transport, outdoor activities), home and work environment (urban vs rural).

Pollution exposure can be estimated by models generated from static measurements, or measured by pollution monitors.

1.5.1. Air pollution monitoring networks

Air pollution monitoring networks are established in most urban areas worldwide – London has one of the world's biggest networks (the London Air Quality Network (LAQN)), with nearly 100 monitors scattered across the city [98] (figure 1.9). Pollutant concentrations are measured continuously, providing data on temporal and seasonal variabilities of air pollution. These data are sometimes used to model the population's external exposure to air pollution – using the mean concentration over a defined time period, with the assumption that exposure remains the same for all inhabitants in the area over the course of the day [99].

Since busy roads contribute to most of London's pollution hotspots, roadside monitors are usually mounted on lampposts or road signs within 1-5 m of busy roads, aimed to not only give an estimate of the public's exposure to air pollutants, but to also identify traffic hotspots [98].

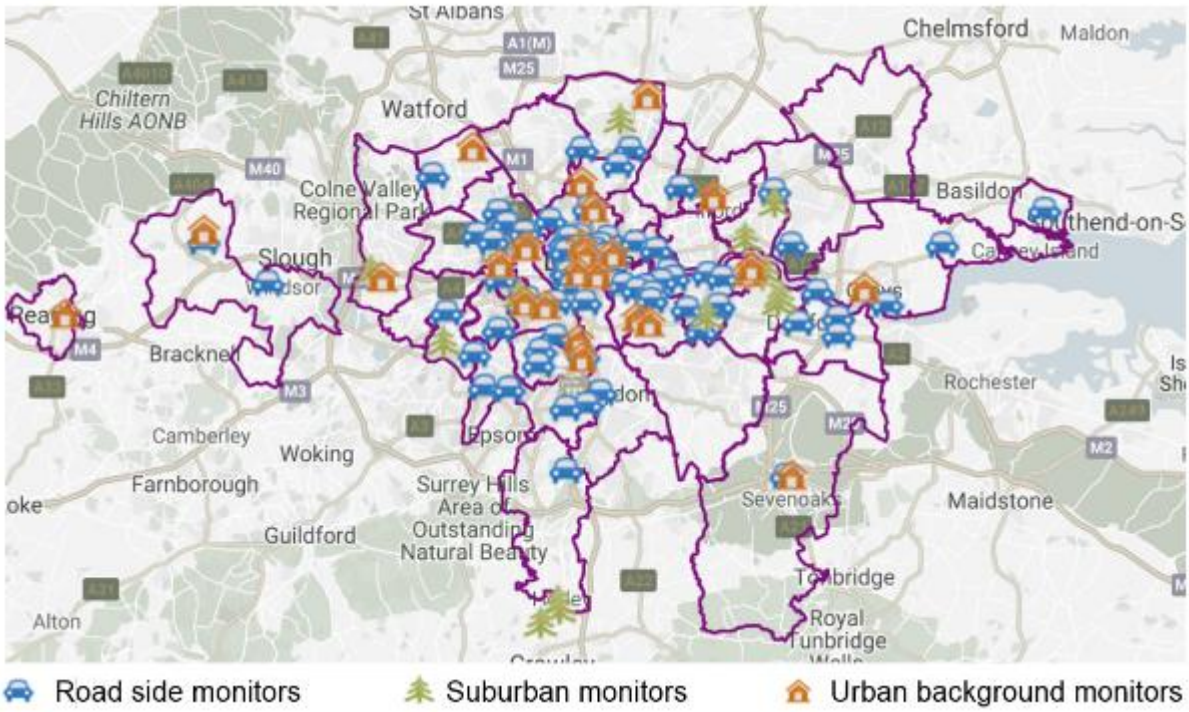

Figure 1.9. London Air Quality Network monitoring sites, showing locations of road side, suburban and urban pollution monitors, measuring carbon monoxide, nitrogen dioxide, ozone, particulate matter ($PM_{2.5}$ and PM_{10}), and sulphur dioxide.
Adapted from the London Air website (https://www.londonair.org.uk/london/asp/publicdetails.asp?region=0).

The LAQN website provides real time and annual information on air pollution in London and south east England. The data are readily available for the general public. Figure 1.10 is an example of real time air pollution map using postcodes. Figure 1.11 is a map showing modelled annual mean NO_2 level in London in 2016.

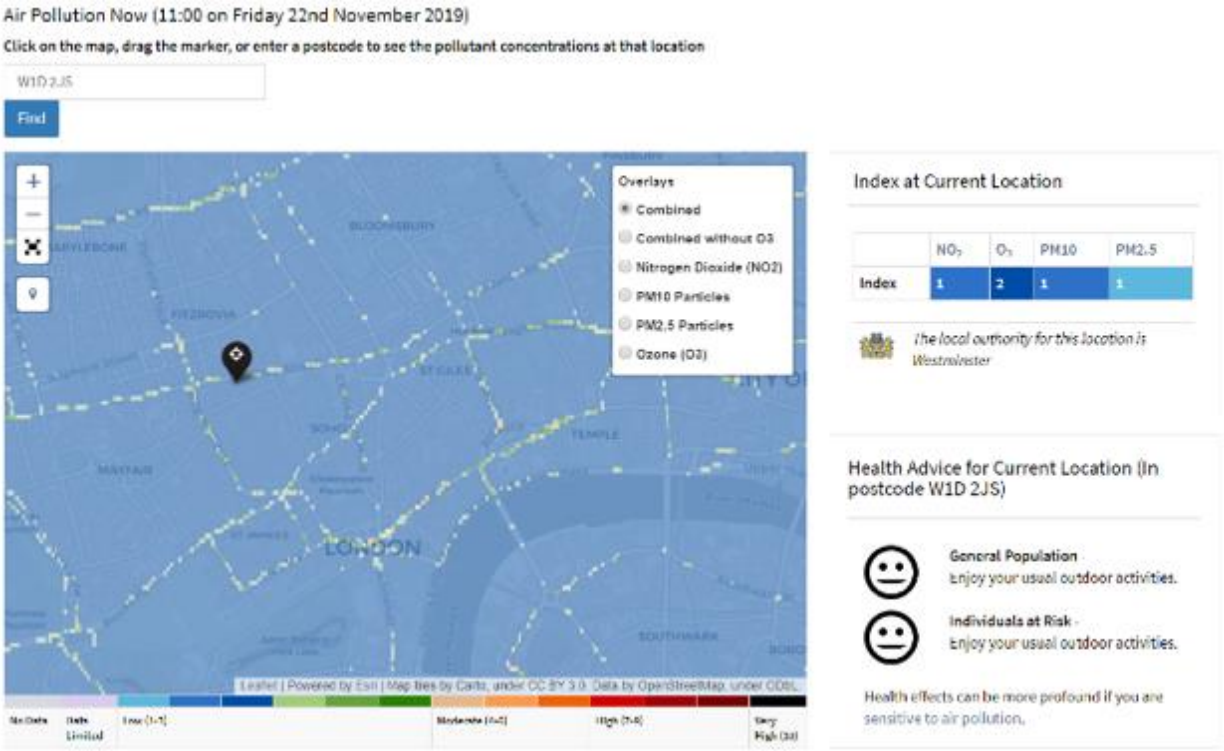

Figure 1.10. Real time air pollution map, showing indices of nitrogen dioxide (NO_2), ozone (O_3), particulate matter (PM_{10} and $PM_{2.5}$), modelled using postcode. Adapted from the London Air website (https://www.londonair.org.uk/LondonAir/Default.aspx).

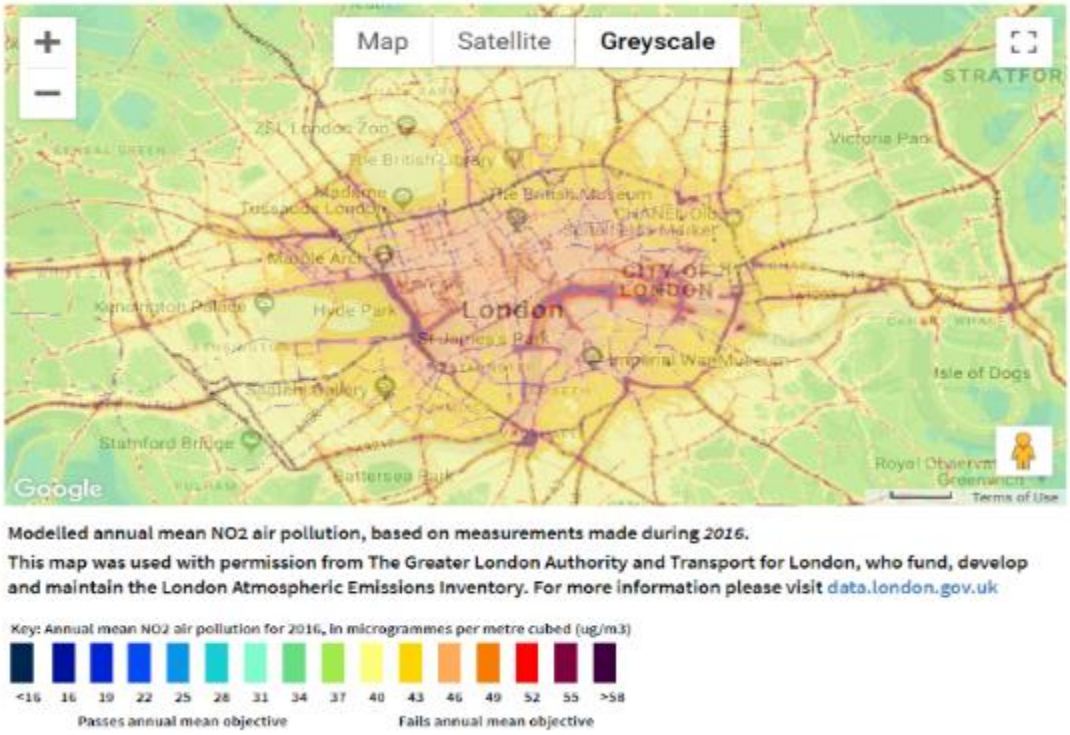

Figure 1.11. Modelled annual mean nitrogen dioxide (NO_2) level in London, using measurements from 2016**.** Adapted from the London Air website (https://www.londonair.org.uk/london/asp/annualmaps.asp).

The advantage of using modelled data on air pollution exposure is that it is easy and ready-made. The disadvantages being it is difficult to separate background from local levels of pollutants; modelling assumes uniform exposure to the population so unless the individual resides closely to a monitor, it may not be an accurate representation of exposure.

1.5.2. Portable monitors

Portable monitors are designed to measure short-term personal exposure to air pollutants more accurately than roadside monitors, without the need to assume equal temporal, spatial and personal factors across the population.

1.5.2.1. Aethalometer

An aethalometer (figure 1.12) is an instrument which measures the concentration of optically absorbing suspended black carbon in a gas stream, as seen in polluted ambient air. Ambient air is forced through the instrument by suction, depositing any black carbon on the filter. A light beam projected through the filter is attenuated by the deposited particles, which are absorbing “black”, since black colour is the most optically absorbent. Measurements are made at successive regular time intervals. The advantages of aethalometers include their pocket-size so they can be worn on person and provide black carbon concentrations in real time, allowing for exposure monitoring in different micro-environments (e.g. commute, outdoor activities, indoor at home or at school). Their use, however, is limited by high cost and battery life – each full battery charge will last approximately 24 hours. This will be discussed in more detail in chapter 2.

Figure 1.12. A portable aethalometer with a black sampling suction tube, measuring the concentration of black carbon in ambient air in real time.

1.5.2.2. Gas diffusion samplers

Gaseous pollutants, such as NO_2 and SO_2, can be collected onto a sampler (figure 1.13) using molecular diffusion technique, measuring their concentrations over time. The advantages include the lower price and ease to use – they are not electrical and can be clipped onto an individual's clothing during the sampling period. The disadvantages are that they are unable to give real time results; the results are derived using multifaceted calculations based on estimated temperature and atmospheric air flow; more details will be discussed in chapter 2. Besides, the samplers are clipped onto clothing or schoolbags, and are easily damaged or misplaced.

Figure 1.13. Nitrogen dioxide diffusive sampler, manufactured by IVL Swedish Environmental Research Institute. The sampler can be clipped onto clothing. NO_2 molecules are quantitatively collected onto a filter behind the silver mesh, which prevents turbulent diffusion, giving a concentration value of NO_2 integrated over time.

1.5.3. Alveolar macrophage black carbon

As mentioned in section 1.2.2., inhaled carbonaceous particles reaching the distal airways are phagocytosed by alveolar macrophages, found along the epithelial surface [100]. The surface area of black carbon visible in AM – alveolar macrophage black carbon (AMBC) – can be measured using light microscopy image analysis. AMBC has been shown to have a dose-dependent relationship with lung function and asthma severity in children [101,102], and is universally accepted as an internal marker which reflects an individual's cumulative exposure to combustion-derived PM [103]. The average clearance half-life of AMBC is reported to be 53 to 116 days, subject to the carbon load [104].

AMs can be sampled by sputum induction or bronchoalveolar lavage (BAL). While BAL is likely to sample macrophages from the alveoli and distal airway, sputum induction may amass macrophages from both lower and upper airway. As mentioned in section 1.2.2.2.1, airway macrophages are thought to be AMs which have travelled upwards along the bronchial tree, therefore they have similar phenotype and functional capacity – sputum induction is therefore accepted as a method of AM sampling. Tillery *et al*.[105] have shown that AMs obtained by sputum induction are larger than those obtained by BAL, containing a higher carbon load. However, once the macrophage size is normalised, AMs obtained by both methods have similar BC phagocytic ability, suggesting both forms of collection are comparable when assessing AMBC. However, BAL is more invasive and requires general anaesthesia in children, while sputum induction carries low risks and can be performed outside of clinical settings. The use of sputum induction to sample AMs will be further discussed in sections 2.6. and 3.5.

1.5.4. Urinary black carbon

A different internal marker for black carbon has been used in recent years – systemic carbon cleared from the circulation can be detected in urine, using white-light generation under laser illumination. Urinary black carbon has been shown to reflect medium to long term exposure to PM [106], and is a non-invasive monitoring method. However, there is risk of contamination from ambient air black carbon during urine collection. Besides, urinary carbon does not exclusively reflected inhaled dose of black carbon which can enter the body through other routes (e.g. gastro-intestinal tract).

Summary: Monitoring individual's air pollution exposure

- Air pollution exposure can be measured either on a population level, using stationary monitors within a national network; or on an individual level using portable monitors.
- Internal biological markers of air pollution exposure are available – alveolar macrophage black carbon loading reflects an individual's long term exposure to particulate matter, albeit relying on the functional capacity of alveolar macrophages.

What is still unknown?

- There is limited data on individual's exposure within different micro-environments (e.g. indoor at school/work, indoor at home, commute to/from school/work, outdoor activities).
- Data on individual's long term personal exposure to pollutants is lacking.

1.6. Protection against air pollution

Since the main sources of air pollution are from fossil fuel combustion and road traffic, policy makers for national and international regulations hold the main responsibility to reduce overall air pollution for the benefit of the general public. However, raising individuals' awareness, and taking various measures to reduce one's exposure to pollution will still be beneficial, particularly for children with chronic respiratory conditions.

1.6.1. Health advice

Information on air pollution is readily available on the internet. WHO publishes guidelines and reports on air pollution regularly; the UK Department of Environment, Food and Rural Affairs website has an air quality forecast; the London Air Quality website provides real time air pollution data across the capital. The British Lung Foundation provides recommendations on various actions to take at different pollution levels (table 1.2). However, these measures cannot provide complete protection, and there is limited information on whether these are attainable over long periods of time in order to achieve health benefits.

Pollution level	Measures
Low	➢ Avoid spending long periods of time along busy roads
Moderate	➢ Reduce or avoid strenuous outdoor activities
High	➢ Reduce or avoid strenuous outdoor activities ➢ Avoid pollution hotspots ➢ Avoid rush hours, travel before pollution levels build up ➢ Use less polluted routes when cycling, walking or running ➢ Use of reliever inhaler if pollution is a trigger to asthma ➢ ± Seek medical attention

Table 1.2. Recommendations on measures to take according to air pollution levels. Adapted from the British Lung Foundation website (https://www.blf.org.uk/support-for-you/air-pollution/tips).

1.6.2. Measures to reduce air pollution exposure

Based on information from the abovementioned organisations, the public are advised to avoid busy roads and pollution hotspots. On an individual level, active forms of transport such as walking, cycling, and scooting are encouraged. Carpooling or use of public transport are more fuel-efficient, and can reduce individuals' contribution to air pollution emissions.

Adequate ventilation during cooking can lower indoor air pollution exposure, especially in houses using gas cook stoves. Air purification is a contemporary solution to indoor air pollution, however even high efficiency air purifiers have limitations. Pollutants may settle before reaching the filter; gaseous pollutant filters have short lifespans and require regular replacement; and ambient air humidity can affect purifiers' performances. Besides, air purifiers rely on electricity, which may not come from sustainable sources.

There are numerous facemasks which claim to reduce inhaled dose of air pollutants, their effectiveness is tested in a laboratory rather than real-life setting. To date, there are limited studies on the biological effectiveness of these masks; their actual health benefits remain unclear.

On a national level, tree and hedge plantations around playgrounds and schools may reduce children's exposure to PM – these act as a physical barricade to intercept airborne particles, but will not remove the presence of such particles.

Tougher governmental regulations on vehicle emissions and scrappage schemes are effective ways to improve ambient air quality. For example, the Institute for Public Policy Research (IPPR) predicted that phasing out diesel engines in London would lead to enough NO_2 reduction to fulfil EU standards [107]. They estimated that 1.4 million life-years could be gained, and up to £800 million financial benefits could be achieved with a 56% reduction in NO_2 and a 45% reduction in NO_X [107].

Summary: Health advice on air pollution exposure reduction

- On an individual level, the public are advised to avoid pollution hotspots, and to engage in activities (e.g. walking, cycling) that contribute less to air pollution generation.
- Online resources are available to advise the public, especially patients with chronic diseases, on measures to take to reduce air pollution exposure.
- On a national level, stricter policies on vehicle emissions and fuel choices are urgently required.

What is still unknown?

- There is limited evidence base for whether the air pollution exposure reduction measures, especially those on an individual level, are achievable over the long periods of time, or sufficient to significantly improve health.
- The biological effectiveness of facemasks remains unclear.

1.7. Cystic fibrosis

In section 1.3., the negative health impact of air pollution was discussed. Children, particularly those with existing respiratory conditions such as cystic fibrosis, are especially vulnerable.

Cystic fibrosis (CF) was first formally described as a disease in 1930s, but references to CF date back to the medieval times when Northern European folklore cautioned *"woe is the child who tastes salty from a kiss on the brow, for he is cursed, and soon must die*".

Cystic fibrosis is the most common hereditary life limiting respiratory condition in the Caucasian population, affecting around 1 in 2500 live births [108]. It is inherited in an autosomal recessive fashion, caused by mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, located on the long arm of chromosome 7. CFTR is a 1480-amino-acid-long transmembrane glycoprotein, an ATP-binding cassette (ABC) transporter-class anion (chloride and bicarbonate) channel protein, which is found in the apical membrane of epithelial cells and submucosal glands [109-111]. The airways have one of the highest expressions of CFTR [112], which also regulate epithelial sodium channel [111]. Over 2000 CFTR mutations have been identified but not all result in CF [113], with some extremely rare variants of unknown functional significances.

Mutations lead to absent (class I), misfolded (class II), defective gating (class III), defective conduction (class IV), insufficient (class V), or unstable (class VI) CFTR (figure 1.14), resulting in defective ion transport, enhanced sodium absorption,

increased sweat sodium chloride concentration, and dysfunctional exocrine glands within the airways, pancreas, liver, reproductive tract and bowel [109]. Consequently, secretions from these organs are more viscous, increasing the chance of ductal blockage [110], while also encouraging bacterial growth.

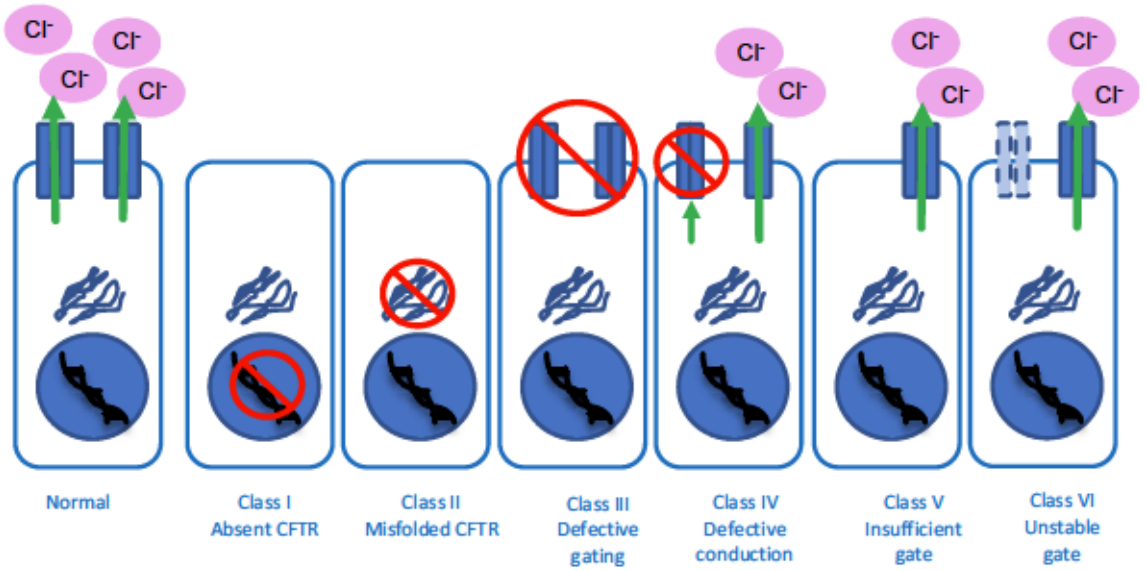

Figure 1.14. Classes of CFTR mutations compared to normal. Class I mutations have no protein synthesis. Class II mutations have impaired protein trafficking due to incomplete folding. Class III mutations have defective channel gating – CFTR reaches cell surface, but does not exhibit gating function. Class IV mutations have defective channel conduction. Class V mutations have reduced protein maturation and therefore less channels. Class VI mutations have unstable protein.

Despite being a multi-organ disease, morbidity of CF is predominantly secondary to chronic obstructive lung disease. Besides the presence of viscous mucus in the airway, it has been suggested that CFTR mutation is associated with abnormal development of cartilaginous airways, thus predisposing individuals to early airflow obstruction [111]. Viscous respiratory secretions predispose patients with CF to pulmonary infections. Not only do recurrent chest exacerbations negatively impact on patients' quality of life, they ultimately lead to end-stage respiratory failure, which is a leading cause of death in this cohort [114].

1.7.1. Host defence in cystic fibrosis

Since ion transport is coupled with water flow, dysfunctional chloride channels will lead to reduction and dehydration of the ASL [115], and subsequent mucociliary functional impairment, allowing harmful inhaled particles and pathogens to persist within the airways [116]. Accumulation of thickened mucus in the lung results in chronic cough, wheeze, recurrent respiratory infections and airway inflammation; the combination of which instigate lung function decline and irreversible lung damage [117].

Normally, the ASL layer is able to initiate rapid bacterial killing. However, a porcine model has shown that a lack of functional CFTR can result in reduced ASL eradication of bacteria, even with relatively little infection or inflammation [118]. This can potentially be explained by the lower pH in CF ASL, which can attenuate anti-microbial peptides such as lysozyme [111]. The low pH is caused by defective bicarbonate transport, which is partly responsible for the production of viscous mucus [111]. Moreover, it has been shown that mice with ASL depletion could develop spontaneous airway inflammation [119], suggesting mucus plugging alone might be significant – potentially due to airborne irritants, such as air pollutants, being trapped in the airways, causing pro-inflammatory cytokines release.

The thick mucus and dehydrated environment of the CF airway can affect the phenotype of macrophages, altering their ability to phagocytose and regulate inflammation [120]. Indeed, studies have suggested that other than epithelial cells, CFTR is also expressed in immune cells such as neutrophils and macrophages –

CFTR in AMs help maintain phagosomal pH, its mutations can therefore lead to defective bacterial killing [121].

1.7.1.1. Alveolar macrophage function in CF

As discussed in section 1.2.2, AMs are important cells for inhaled PM removal. AMs obtained from BAL samples of children with CF showed reduced phagocytic ability secondary to reduced MHC Class II and mCD14 (a monocyte/macrophage differentiation antigen which plays a role in macrophage TLRs activation by lipopolysaccharide) expression during disease exacerbations [122]. Reduced scavenger receptor expression was seen on AMs obtained from induced sputum of patients with CF, resulting in impaired florescent particle uptake compared to their healthy counterparts [123]. This was supported by findings in a bronchiectasis cohort, where AMs from children with bronchiectasis also had reduced expression of scavenger receptors and dysfunctional phagocytosis *in vitro* [124]. The size of AMs can also influence their function – compared to healthy controls, smaller AMs are often found in patients with CF; these AMs have weaker phagocytic ability when confronted by un-opsonised particles [123]. Interestingly, despite their smaller size, absolute number of AMs found in CF airways is higher during late fetal stage and childhood, even in the absence of active infection [120], suggesting the condition is intrinsically pro-inflammatory – this will be explored further in section 1.7.2.

To better understand AM phagocytic function in CF, a literature search on PubMed was performed. Using the key terms “alveolar macrophage”, “cystic fibrosis”, and “phagocytosis”, searching articles published in English from years 1980-2019, 60 articles were identified, 9 of which were summarised in appendix 1 – excluding

review articles, studies focusing on how bacteria invade or interfere with AM function (rather than looking at CF AM function as a primary objective), and studies not related to cystic fibrosis. From the studies reviewed, CFTR knock-out mice showed reduced phagocytosis of bacteria (e.g. *Pseudomonas aeruginosa* and *Staphylococcus aureus*); human studies using AMs obtained from sputum induction or bronchoalveolar lavage, and human monocyte-derived macrophages, not only showed similar phagocytic impairment, but further demonstrated the suppressive effects of CF secretions. These *in vitro* findings suggest that AM phagocytosis of bacteria, fungi, apoptotic cells and un-opsonised fluorescent particles is impaired in CF, potentially related to the altered airway environment that the cells are exposed to.

1.7.1.2. Other host defence impairment in cystic fibrosis

The altered CF immune system is undoubtedly multifactorial. Aside from the aforementioned mechanisms, other immune pathways are also affected. For example, not only is CFTR involved in neutrophil development, its mutation can also disturb neutrophil conductance of halides across the phagosomal membrane, thereby reducing their bacterial-killing capacity [125]. CFTR is also responsible for maintaining expression of many genes which are implicated in important immune processes, such as phagocytosis, complement activation, T-cell and B-cell activation, and apoptosis; suggesting CFTR has alternative roles to chloride transportation [126].

1.7.1.3. Opportunistic infections in cystic fibrosis

With an altered immune system, children with CF are susceptible to opportunistic infections; pathogen colonisation is encouraged by viscous mucus and impaired mucociliary clearance. An example is *Pseudomonas aeruginosa*, which is isolated in 80% of patients' airways [127], contributing substantially to morbidity and mortality. *P. aeruginosa* is able to form biofilms, express virulence factors in order to resist the innate immune system, and adapt and mutate during chronic infections [128]. Despite the hypoxic environment of CF airways due to increased oxygen consumption by the epithelium [129], the growth of *P. aeruginosa* can occur through anaerobic means using nitrate and nitrite, both are found within CF respiratory secretions [130]. Other pathogens commonly isolated in CF airways include *Staphylococcus aureus, Haemophilus influenzae, Stenotrophomonas maltophilia, Achromobacter xylosoxidans,* non-tuberculous mycobacteria and *Burkholderia cepacia,* all contributing to recurrent chest exacerbations and lung function deterioration.

1.7.1.4. Mechanisms of bacterial survival within the host

Comparing macrophages with normal vs defective CFTR showed that the autophagy genes were downregulated in CFTR defected cells, resulting in an increased amount of bacteria present intracellularly [131]. Indeed, there is evidence that some organisms are able to invade and survive intracellularly in patients with CF via two ways: 1) cytosolic bacteria can avoid endocytosis, and replicate within the host cytosol; 2) intravacuolar bacteria can reside and replicate within the host vesicular endomembrane system, inside a modified membrane-bound

compartment [132]. Remarkably, despite being key immune cells, macrophages seem to be targeted by these intracellular bacteria as a place of residence.

An example of such bacteria is *Burkholderia cepacia*. With resistance to multiple antibiotics, it renders the host ineligible for lung transplantation and presents a major challenge in CF management. It has been shown that *Burkholderia* species are able to enter, survive, invade and replicate intracellularly within macrophages [133], where they grow as single or clusters of cells [134].

Other than CF, bacteria-laden macrophages can also be found in other chronic inflammatory diseases such as inflammatory bowel disease. These macrophages express higher levels of cytokines compared to un-laden macrophages, thereby contributing to and worsening the chronic inflammation cascade [135]. Indeed, high amounts of cytokines are present in the CF airway – this will be further discussed in the next section.

1.7.2. Airway Inflammation in cystic fibrosis

CF lung disease is predominantly secondary to recurrent bacterial infections and chronic airway inflammation, inevitably resulting in respiratory decline [116]. As mentioned in section 1.7.1.1., while recurrent infections were traditionally accepted as promoters for inflammatory cascades, studies have demonstrated that an intrinsic pro-inflammatory state exists in the CF airway even without evidence of infection during the fetal stage [136]. The overwhelming presence of inflammatory cytokines and prostaglandins within the CF airway contributes to inflammation [137] and lung damage. This pro-inflammatory environment can also promote

macrophage polarisation to M1 macrophages [120]; while dysfunctional CFTR can affect polarisation to M2 macrophages – this imbalance of M1 and M2 macrophages plays a part in the excessive inflammatory response in CF [31]. There is also evidence that peripheral macrophages from CF patients have an amplified inflammatory response to mediators, suggesting that CF macrophages are intrinsically pro-inflammatory [120].

Upon infections, airway cells release inflammatory mediators which stimulate neutrophil influx. While neutrophils are recruited to fight pathogenic organisms, they also pose detrimental effects. Neutrophil influx leads to the release of oxygen radicals and proteases such as elastase. This, coupled with overwhelmed anti-elastase defence, result in structural lung damage [116,138,139]. Indeed, airway neutrophil elastase activity detected in early life of children with CF was shown to be related to the development of bronchiectasis in childhood [112].

1.7.2.1. Prostaglandins and cyclooxygenase in cystic fibrosis

It has been suggested that defective CFTR is associated with altered regulation of inflammation. For example, prostanoid metabolism in the lower airways in CF is abnormal, causing an overproduction of prostaglandins (PGs) [140]. This can theoretically explain the presence of inflammation despite a lack of infection in early neonatal life.

PGs, leukotrienes (LT) and lipoxins are all eicosanoids. PGs are lipid compounds that regulate inflammatory responses such as vasoconstriction or dilatation, inflammatory cell chemotaxis, platelet aggregation and pain sensitisation; they play

an important part in lung diseases such as asthma and CF. PGs are produced by most nucleated cells, and are released in the airways from epithelial cells, DCs, fibroblasts and AMs. Types of PGs include prostaglandin D2 (PGD_2), prostaglandin E2 (PGE_2), prostaglandin F2α ($PGF_{2\alpha}$), and prostaglandin I2 (PGI_2) – these subtypes have different roles and bind to different E-prostanoid receptors (EP_1-EP_4). For example, PGD_2 is known to play a role in the development of atopy; while PGE_2 acts mostly on the smooth muscles of the respiratory and gastrointestinal tracts, mediating inflammation through ligation of G protein-coupled receptors [141].

Arachidonic acid (AA), a fatty acid derived from diet, is metabolised by cyclooxygenase (COX) to form PGs (figure 1.15).

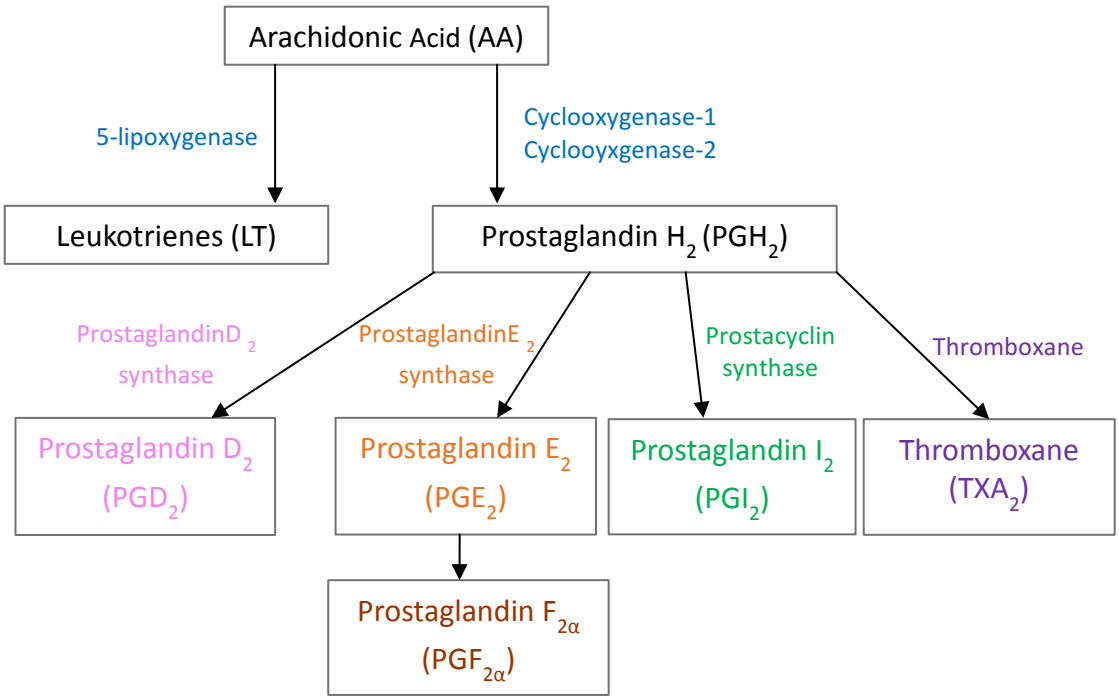

Figure 1.15. Prostaglandin synthesis pathway.

COX are classified into COX-1 and COX-2. COX-1 enzymes are found in most tissues and are involved in PG production under physiological conditions.

Conversely, COX-2 enzymes are rarely found in tissues, they act to produce PGs only when induced by inflammatory stimulants [142]. COX-2 is therefore regarded as an anti-inflammatory target. Indeed, in a human CF bronchial epithelial cell line, Chen *et al.* [137] demonstrated that COX-2 expression was increased with over-production of PGE_2; they also showed similar findings in CFTR knockout mice (figure 1.16). Roca-Ferrer *et al.* [140] showed that COX-2 was up-regulated in nasal polyps in patients with CF, leading to increased production of PGs (figure 1.17). CFTR is therefore proposed to play a role in AA metabolism – as a protective regulator of the inflammatory response mediated by PGE_2 [143]. Certainly, increased levels of PGE_2 are seen in BAL, sputum, exhaled air, saliva and urine of patients with CF [144-148].

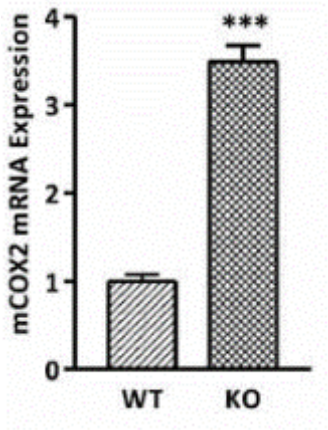

Figure 1.16. Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) results showing higher levels of cyclooxygenase-2 (COX-2) mRNA in the lungs of CFTR −/− (KO) mice compared to CFTR +/+ (WT) mice (***$p < 0.001$). Reproduced with permission from Chen *et al.* [137], Journal of Cellular Physiology.

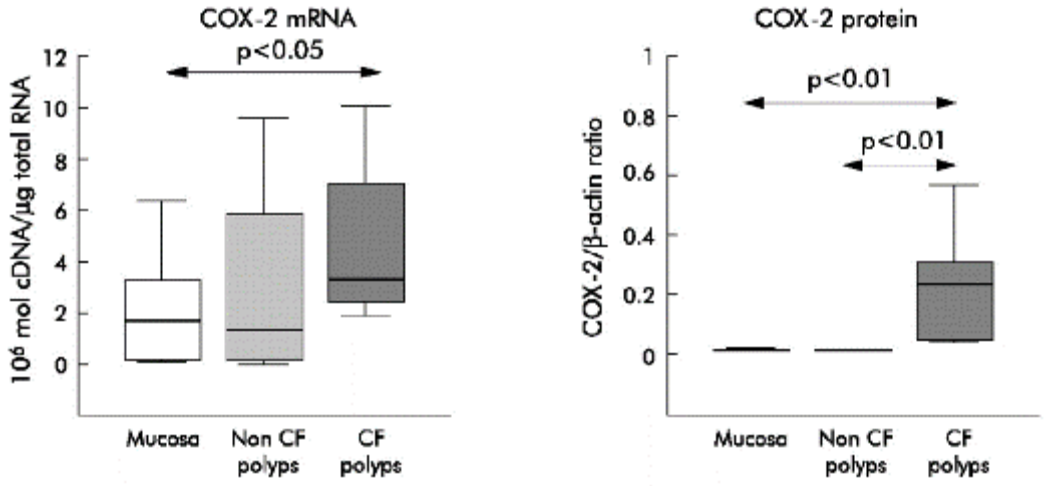

Figure 1.17. Reverse transcription polymerase chain reaction (RT-PCR, 10^6 cDNA molecules/µg total RNA) of cyclooxygenase-2 (COX-2) in CF nasal polyps, non-CF nasal polyps, and nasal mucosa. Reproduced with permission from Roca-Ferrer *et al.* [140], Thorax.

Of all PG types, PGE_2 is reported to have an inhibitory effect on AM functions. In the airway, production of PGE_2 takes place in epithelial cells, endothelial cells and leukocytes including AMs, neutrophils, T cells and dendritic cells – with AMs being a dominant source during infections [149]. Using rat AMs, PGE_2 has been shown to inhibit microbicidal activities and phagocytosis of apoptotic cells [141] (figure 1.18). It can also inhibit phagocytosis of urban particulate matter [102] (figure 1.19)

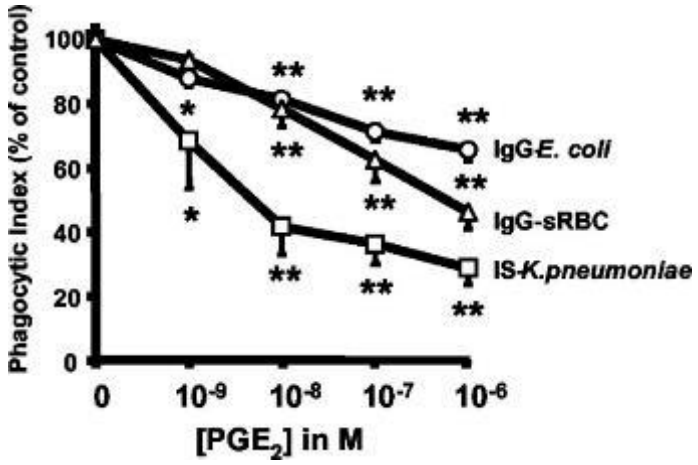

Figure 1.18. Inhibition of rat alveolar macrophage phagocytosis of IgG-opsonised sheep red blood cells (sRBC), immune serum (IS)-opsonised live *K. pneumoniae*, and FITC-labelled IgG-opsonised *E.coli*; when exposed to different concentrations of PGE_2. Data showing the inhibitory effects of PGE_2 on phagocytic ability. Reproduced with permission from Aronoff *et al.* [141], The Journal of Immunology.

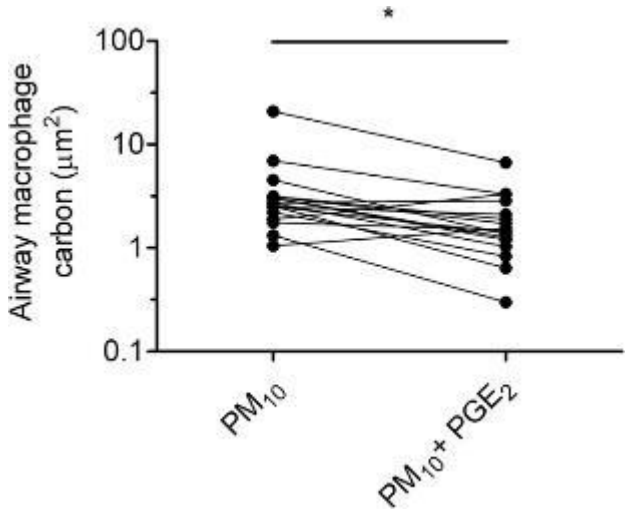

Figure 1.19. Effect of PGE_2 on phagocytosis of urban PM_{10} by rat airway macrophages. Data demonstrating the inhibitory effect of PGE_2 on AM phagocytosis (*p<0.05). Reproduced with permission from Brugha *et al,* Thorax.

Acting via inhibition of TNF-α and IL-12 production, and reduction of MHC II expression, PGE_2 can suppress proliferation, differentiation, migration, and activation of macrophages at a concentration as low as 10^{-10} M [150]. This suppressive effect is associated with increased intracellular cyclic adenosine monophosphate (cAMP) production on ligation of receptors EP2 and 4 [141,151]. EP2 receptor activation prohibits FcR-mediated phagocytosis [149]; and is most frequently expressed on maturing macrophages. It is therefore considered the major mediator in the inhibitory effect of PGE_2 [150]. Unsurprisingly, the inhibitory effect of PGE_2 can be reversed by EP2 antagonists [141] (figures 1.20 and 1.21).

Figure 1.20. Left: PGE_2 acting on a macrophage via EP2 receptors. Right: EP2 antagonist blocking EP2 receptors, PGE_2 therefore unable to act on macrophage.

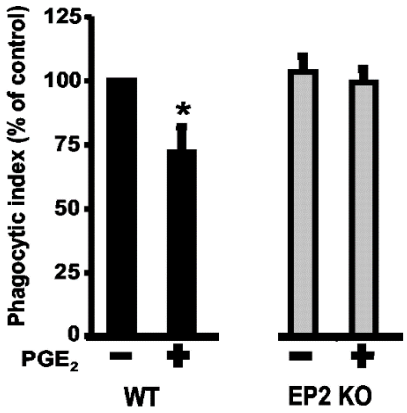

Figure 1.21. Effects of PGE_2 on AM phagocytosis in wide type (WT) and EP2 knock-out (KO) mice, when exposed to opsonised *E.Coli*. Data showing EP2 KO mice are protected from the inhibitory effect of PGE_2 on phagocytosis. Reproduced with permission from Aronoff *et al.* [141], The Journal of Immunology.

Since PGs are chemically unstable, urinary metabolites of PGs are generally accepted as an index of PG biosynthesis. Notably, concentrations of PGE_2 metabolites are shown to be associated with genotype severity in CF [143].

In order to better understand the interactions between PGE_2 and AM function, a literature search on PubMed was performed, using the key terms "PGE_2", "alveolar macrophage" and "phagocytosis". Searching articles published in English from years 1980-2019, 52 articles were identified, 9 of which were reviewed, excluding review articles and studies unrelated to PGE_2 or AM function. A summary of these studies can be found in appendix 2. All reviewed studies suggest that PGE_2 can inhibit macrophage function, including bacterial, viral or fungal clearance and killing. Of note, increased production of PGE_2 is seen in post bone marrow transplant murine models, resulting in impaired host defence against bacteria. One animal model showed that pre-treatment with COX inhibitor could reverse AM phagocytosis suppression. Despite strong associations between PGE_2 and impaired AM

phagocytosis, studies using primary human AMs to assess the effects of PGE_2 are lacking.

Despite previous work showing both PGE_2 and PGD_2 supressing human monocyte-derived macrophage phagocytosis (figure 1.22), albeit PGD_2 to a lesser extent than PGE_2 [152], other studies have shown that PGD_2 enhances and activates macrophage effector function [153,154]. Therefore, PGE_2 remains a major focus as a suppressor of AM function.

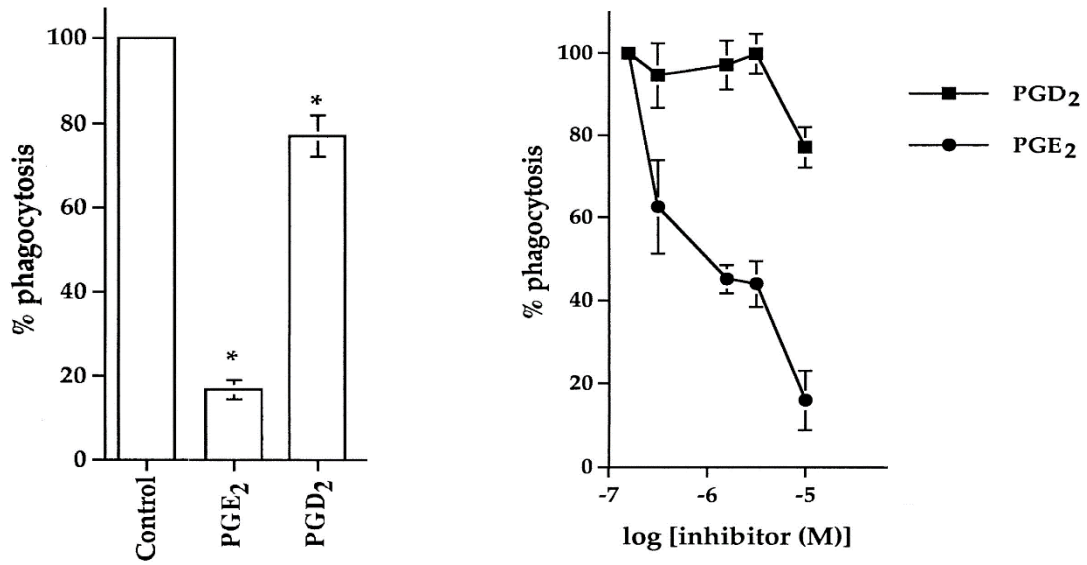

Figure 1.22. Left: Human monocyte-derived macrophages pretreated with PGE_2 (10 μM) and PGD_2 (10 μM) for 15 min, assessed for *in vitro* phagocytosis of cultured neutrophils. The percentage of macrophages ingesting apoptotic neutrophils is expressed as the mean ± SEM. *$p < 0.05$ compared with control. Right: Concentration-response effects of PGE_2 and PGD_2 on macrophage recognition of apoptotic cells. Human monocyte-derived macrophages pretreated with varying concentrations of PGE_2 and PGD_2 for 15 min, assessed for *in vitro* phagocytosis of cultured neutrophils. The percentage of macrophages ingesting apoptotic neutrophils is expressed as the mean ± SEM. Reproduced with permission from Rossi *et al.*[152], the Journal of Immunology.

1.7.2.2. Cytokines in cystic fibrosis

The CF airway is overwhelmed with inflammatory cytokines. These are secreted by epithelial cells, macrophages and neutrophils; secondary to COX-2 expression [155] and CFTR dysfunction [117]. As mentioned above, structural and disease progression in CF may be independent of bacterial infections [156], signifying that intrinsic inflammatory processes occur intuitively even without external insults or triggers.

Upon pathogenic invasion, phagocytes, including macrophages, eliminate and maintain inflammation by producing tumour necrosis factor (TNFα), and interleukin (IL-1β, IL-6, IL-8, IL-12) [37,157], all known to be elevated in CF lungs [120]. With macrophages being the first responders to invasion, they are likely to be the first to release cytokines such as IL-8, which subsequently recruit neutrophils [37]. Indeed, IL-8 is found to be increased in respiratory secretions of CF patients even during stable clinical states [158].

1.7.3. Treatments and therapies in cystic fibrosis

Life expectancy of CF has improved dramatically over the last few decades. In 2011, the median survival was 41.4 years, but patients born after 2000 are estimated to survive beyond 50 years [159].

Traditional treatments of CF include airway clearance with nebulised mucolytics (e.g. hypertonic saline, dornase alfa), inhaled bronchodilators (e.g. salbutamol), topical nasal steroids for polyps, and chest physiotherapy. Chest exacerbations are managed with antibiotics which are usually empirical but can also be tailored according to sputum or cough swab microbiology growths. Special dietary

requirements in CF include pancreatic enzymes (e.g. Creon) with meals, vitamins and mineral supplements, and in some cases, high calorie supplements. In severe cases of CF, lung transplantation will be considered. This is a complex process requiring meticulous planning, and candidate selection can be challenging. The timing of listing patients for transplant is crucial, usually guided by lung function and respiratory complications such as exacerbations, pneumothorax and haemoptysis. Contraindications such as infections with resistant organisms such as *B. cepacia*, also need to be considered.

A more recent development in CF treatment is CFTR modulators which restore or improve mutant CFTR function. To date, three CFTR modulators have been approved for use in CF – ivacaftor (a “potentiator” for gating mutations – e.g. G551D – by opening the defective chloride channels), lumacaftor/ivacaftor (a combination treatment with a “corrector” which enables correct CFTR formation and trafficking to cell surface, and a “potentiator”) and tezacaftor/ivacaftor (another combination of “corrector” and “potentiator”). Presently, triple drug combination therapies are undergoing clinical trials with the potential to enhance overall clinical effectiveness. Although promising as the CFTR modulators appear to be in clinical trials, many patients are not able to receive these treatments. Lumacaftor/ivacaftor was previously not recommended by the National Institute for Health and Care Excellence (NICE) guidelines due to funding; while the NICE review on tezacaftor/ivacaftor was suspended. It was only recently that these drugs became available on the National Health Service in the UK. Beside the availability of these drugs, there are also patients with mutations that are not targeted by the modulators. Therefore, while new treatments should be explored and developed, management

of disease complications (e.g. viscous mucus, recurrent infections, inflammation and chest exacerbations) should remain a priority in order to improve patients' quality of life.

In relation to the discussion in section 1.7.2.1., COX inhibitors such as non-steroidal anti-inflammatory drugs (NSAID, e.g. Ibuprofen), when used in high dose (defined as peak serum concentration of 50 to 100 µg/ml, daily dose adjusted accordingly for each individual), were shown to slow lung disease progression in patients (adult and paediatric) with CF with overall good tolerance [160,161] (figure 1.23). This was supported by a recent paediatric cohort study which showed that high dose Ibuprofen was associated with slower lung function deterioration and improved survival in children with CF [162].

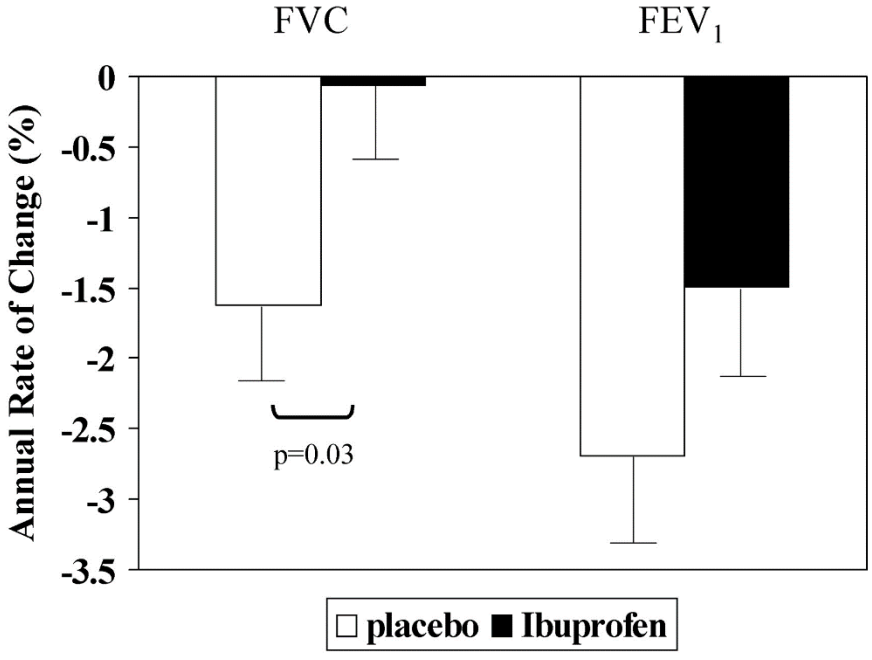

Figure 1.23. Annual rate of change in lung function between ibuprofen and placebo groups: forced vital capacity (FVC) % predicted of −1.62% ± 0.52% for placebo vs −0.07% ± 0.51% for ibuprofen. No significant difference in mean annual rate of decline in forced expiratory volume in 1 second (FEV_1) % predicted. Reproduced with permission from Lands et al. [157], the Journal of Pediatrics.

Summary: Host defence in cystic fibrosis

- CFTR mutations result in thickened airway secretions, rendering the hosts susceptible to recurrent bacterial infections, lung function decline and structural damage.
- Alveolar macrophages in CF are smaller in size, with reduced expression of cell surface receptors, and therefore reduced phagocytic capacity.
- CF patients are susceptible to opportunistic infections, not only is the CF airway encouraging to bacterial growth due to increased mucus viscosity, bacteria (e.g. *Burkholderia* species) can survive and replicate within phagocytes.
- There is an imbalance of M1 and M2 macrophage polarisation in CF, which further aggravates the underlying airway inflammation caused by recurrent respiratory infections.
- Prostaglandin mediated inflammatory responses are regulated by CFTR; and there is an increased production of prostaglandins in CF.
- PGE_2 is shown to impair AM phagocytosis in animal models.

What is still unknown?

- There are no current studies looking at AM phagocytosis of air pollutants.
- There is a lack of human studies looking at the role of PGE_2 in AM functional impairment in CF, and the consequences of such dysfunction.
- It is unclear whether PGE_2 is permanently elevated in CF airways.
- The roles of other prostaglandins remain unclear.
- The potential modulation of PGE_2 production, and the benefits of using low dose COX inhibitor in CF remain uncertain.

1.8. Air pollution and cystic fibrosis

With a compromised immune system, children with CF are more vulnerable to the adverse effects of ambient air pollution, which predisposes them to chest exacerbations [163] and subsequent lung function deterioration.

1.8.1. Respiratory infections and pulmonary exacerbations

It has been shown that PM, NO_2 and ozone levels around CF patients' home addresses correlate positively with episodes of chest exacerbations [163] (figure 1.24).

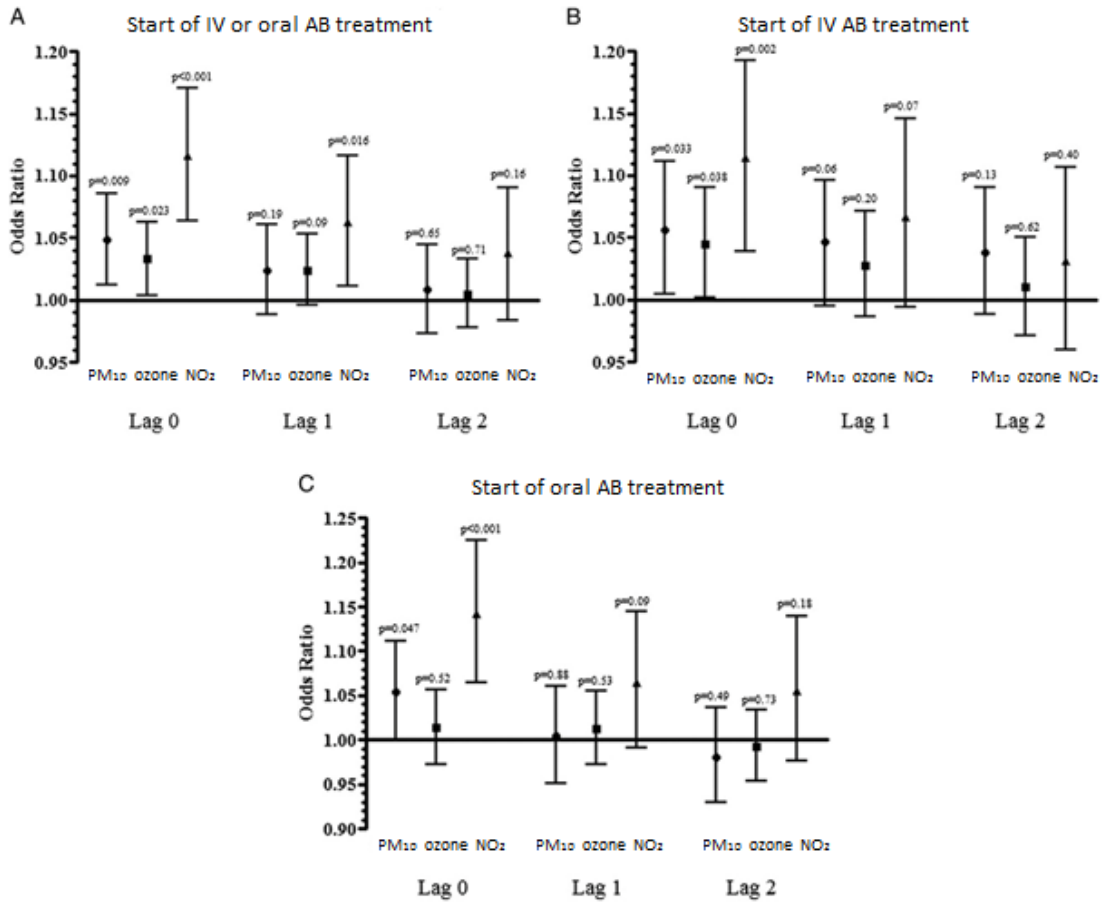

Figure 1.24. Antibiotic (AB) treatment per 10 µg/m^3 increase in PM_{10}, ozone and NO_2. Data showing: (A) Assessment of all events (at the start of either oral or IV treatment): at lag 0, there is an overall significance. At lag 1, only NO_2 showed significance. (B) Assessment of start of IV treatment: at lag 0, there is an overall significance. (C) Assessment of start of oral treatment: at lag 0, there is an overall significance, except for ozone.
lag 0 = the day of the start of antibiotic treatment; lag 1 = the day before the start of antibiotic treatment; lag 2 = 2 days before the start of antibiotic treatment. Adapted and reproduced with permission from Geominne *et al*[163], Chest.

A retrospective study of children aged <5 years by Psoter *et al.* [164] showed that a 10 µg/m^3 increase in $PM_{2.5}$ exposure was associated with earlier *Pseudomonas aeruginosa* acquisition (i.e. 24% increased risk of *Pseudomonas* acquisition). They later demonstrated in another retrospective study of children <6 years of age, that the same increase in $PM_{2.5}$ exposure led to a 68% increased risk of methicillin resistant *Staphylococcus aureus* (MRSA) acquisition during follow up [165], suggesting $PM_{2.5}$ as a risk factor for initial *Pseudomonas* and MRSA acquisition in children with CF.

1.8.2. Lung function decline

Further to section 1.3.2.1., air pollution can impact on children's lung function and growth. These effects will affect children with CF more, because FEV_1 is an important predictor of their morbidity and mortality. Indeed, Goss *et al*. [166] showed that an increase of $PM_{2.5}$ by 10 µg/m^3 could reduce the FEV_1 by 155 ml in patients with CF.

1.8.3. Dysfunctional alveolar macrophage in cystic fibrosis

Functional AMs phagocytose inhaled PM, thereby protecting other airway cells from the inflammatory processes induced by non-phagocytosed PM – this is likely to be attenuated in CF, where phagocyte function is deficient. As reported by Wright *et al.* [123], sputum macrophages from patients with CF are smaller and have reduced MARCO and CD206 (mannose receptor) expression (figures 1.25 and 1.26). Impaired autophagy with altered phagosome maturation, phagolysosome formation, lysosomal function and lysozyme production are seen in CF [120]. This is supported by an animal study which demonstrated impaired nanoparticles uptake

by macrophages in CFTR mutant mice, resulting in increased particle uptake by epithelial cells, and a normal inflammation resolution [167].

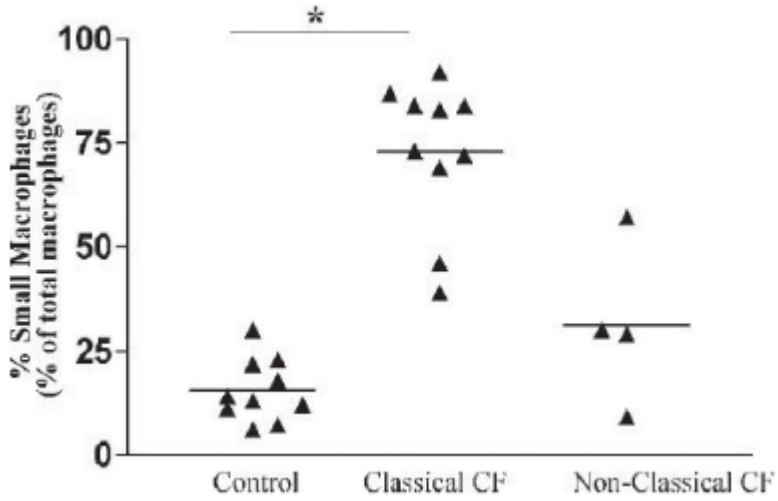

Figure 1.25. Percentage of small macrophages in adult classical CF patients (age 21.5 ± 4 years), non-classical CF patients (age 42.5 ± 10 years), and controls (age 35.2 ± 12 years). The average value for the controls was 16 ± 8%; classical CF was 73 ± 18%, and non-classical CF was 31 ± 20%. * $p < 0.0001$, unpaired *t*–test. Bars represent means. Reproduced with permission from Wright et al. [123], Journal of Leukocyte Biology.

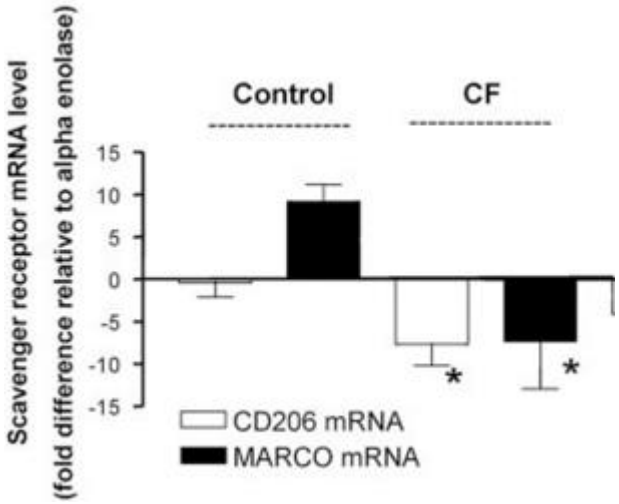

Figure 1.26. Presence of CD206 and MARCO receptors mRNA isolated from control (n = 3) and CF (n = 3) macrophages. * $p < 0.01$. Reproduced with permission from Wright et al. [123], Journal of Leukocyte Biology.

Further to section 1.7.2.1., increased levels of prostaglandin can inhibit AM function. Since CFTR increases PG production, it is reasonable to consider PG as a one of the causes of dysfunctional AM in CF.

In order to obtain a more comprehensive view on the effect of air pollution on cystic fibrosis, a literature review on PubMed for articles published in English from years 1980-2019 was performed, using the key search terms “air pollution” and “cystic fibrosis” identified 89 articles, 12 of these were summarised in appendix 3, excluding review articles, or articles not related to the effects of air pollution on patients with cystic fibrosis. The studies suggest patients with CF are more susceptible to air pollution – exposure to air pollutants can reduce the CF host’s immune capacity to respond to insult, leading to increased oxidative stress, necrosis and cell death. It is also associated with an increased risk of initial bacterial acquisition, antibiotic usage, and pulmonary exacerbation.

1.8.4. The potential speculated mechanisms of adverse effects of air pollutants on patients with CF

1) This chapter has demonstrated that AM phagocytosis is impaired in CF – this could potentially lead to residual carbonaceous PM remaining in the airway. The non-phagocytosed particles may invade other airway cells, such as epithelial cells, propagating the inflammatory cascade, thereby worsening the pre-existing inflammatory state in CF – contributing to chest exacerbations and lung function decline.

The shift of inhaled PM away from AMs to other cells can affect the release of cytokines such as IL-8 from epithelial cells – a study using human bronchial epithelial cells demonstrated that PM exposure encouraged pro-inflammatory cytokine production[168]. On the contrary, another study using a bronchial epithelial cell-line (BEAS-2B) showed that IL-6 and IL-8 secretions were reduced following exposure to realistic doses of gasoline exhaust, suggesting impairment of the epithelial defence mechanisms [169]. Indeed, exposure of CF human bronchial epithelial cells to nanoparticles could enhance necrosis compared to healthy controls, with a dose-dependent rise in cytokines [170], thus potentiating the inflammatory process.

2) PM can promote bronchial epithelial cell apoptosis in CF, via the activation of caspase-9 protein [22]. As a result, particle clearance can be hindered.

3) O_3 can down regulate CFTR transcription in epithelial cells, reducing CFTR-mediated chloride transport [171] – this phenomenon is understandably exaggerated in CF.

4) Air pollution can increase CF patients' susceptibility to respiratory infections, another driver for their respiratory decline. Studies have demonstrated that PM exposure could impede clearance of *P. aeruginosa*, increase *Streptococcus pneumoniae* adhesion to epithelial cells, and alter the biofilm structure of *Staphylococcus Aureus*, thus affecting the response to antibiotic treatments [22].

Summary: Air pollution and cystic fibrosis

- Air pollution exposure can impact on the lung function of patients with CF.
- The immune system is impaired in CF – with altered AM function and abnormal phagocytosis.
- Impaired AM phagocytosis of nanoparticles can lead to increased particle uptake by epithelial cells, as demonstrated by animal models.
- *In vitro* studies suggested air pollutants could cause inflammation and impair epithelial defence mechanism.
- Air pollution exposure can hinder bacterial clearance in CF.

What is still unknown?

- There is a lack of human studies on the mechanisms underlying the vulnerability of patients with CF to air pollution.
- The specific impacts of chronic exposure to air pollution on patients with CF remain unclear.
- There is limited data on the role of prostaglandin in the susceptibility of patients with CF to air pollution.

1.9. Summary

Air pollution is an ongoing and evolving problem in the UK. In high income countries, the main culprit of ambient air pollution is road traffic, with diesel engines being responsible for the majority of air pollutants, such as PM and NO_2 in big cities. Air pollution can affect everyone at every stage of the life course, but children are most vulnerable because of their immature immune system and ongoing organ growth, with lung growth and function being most affected. Children with chronic respiratory conditions are even more susceptible.

In CF, the disease pathogenesis dictates that the immune system is compromised and therefore patients are more affected by air pollution. Thickened airway mucus and impaired mucociliary clearance impede removal of inhaled particles; recurrent respiratory infections and ongoing airway inflammation are further exacerbated by pro-inflammatory insults such as air pollutants; defective AM phagocytosis renders epithelial cells vulnerable to the pro-inflammatory effects of non-phagocytosed PM lingering along the airways. Indeed, air pollution can worsen the outcome in CF – it increases the risks of bacterial infections and pulmonary exacerbations, and subsequent lung function decline. Furthermore, ultrafine non-phagocytosed PM could leach into the systemic circulation and affect distant organs, leading to new onset or exacerbations of other systemic conditions.

Despite clear evidence that patients with CF are vulnerable to the adverse effects of air pollution, to date, human studies underpinning the underlying mechanism of impaired phagocytosis are lacking. However, it is known that AM phagocytosis of bacteria is impaired in CF compared to healthy controls; and that phagocytosis is

attenuated by prostanoids – which are present in higher quantities in CF than the healthy counterparts. It is therefore reasonable to consider prostanoids as a putative explanation for the phagocytosis impairment in CF.

1.10. Research hypothesis and aims

I hypothesised that phagocytosis of black carbon by alveolar macrophages was impaired in CF, contributing to the vulnerability of children with CF to air pollution; and that this was mediated by increased production of phagocytosis-inhibitory prostanoids in the airways.

Since the amount of BC in AMs is used as an internal marker for long-term exposure to air pollution, AMBC can also be used to determine the intrinsic phagocytic ability of AMs, provided that the dose of inhaled PM are relatively constant. I therefore hypothesised that, under the exposure to comparable levels of ambient air pollution, the amount of inhaled BC phagocytosed by AMs *in vivo* would be reduced in CF compared to healthy controls; and that this was secondary to increased production of prostaglandins in patients with CF.

As a result of impaired uptake of PM by AMs in CF, residual PM would affect other airway cells, worsening pre-existing airway inflammation, with the potential for ultrafine particles to leach into the systemic circulation and travel to distant organs. There is robust evidence to support the link between air pollution and adverse fetal and neonatal outcome, but the underlying mechanisms remain unclear. The placenta can be used as a proxy for distant organs – i.e. ultrafine particles not phagocytosed by maternal lung can penetrate the tissue barriers and infiltrate the systemic circulation, translocating to the placental tissues.

1.10.1. Specific hypotheses

In children with cystic fibrosis, alveolar macrophage function is impaired due to upregulation of cyclooxygenase and increased production of prostaglandin (particularly prostaglandin E2) – which can be reversed by cyclooxygenase inhibitor. Impaired macrophage phagocytosis will result in residual inhaled carbonaceous particles in the airway, affecting epithelial cells and aggravating airway inflammation; while residual ultrafine particles may cross tissue barrier and translocate to distant organs via the circulation.

1.10.2. Aims

- To compare personal exposure to air pollution of children with CF and healthy controls.
- To compare *in vivo* alveolar macrophage black carbon between children with CF and healthy controls.
- To assess *in vitro* phagocytic function of alveolar macrophages isolated from children with CF and healthy controls.
- To compare COX-2 expression in alveolar macrophages isolated from children with CF and healthy controls.
- To compare the production of prostaglandins by measuring prostanoids and metabolites in sputum and urine between the two groups.
- To establish the effects of PGE_2 on phagocytosis by alveolar macrophages.
- To assess the possibility of reducing *in vivo* prostanoids production with cyclooxygenase inhibitors.
- To investigate the possibility of epithelial cell invasion by particulate matter in the presence vs absence of functional alveolar macrophages.

- To model whether exposure to black carbon increases release of cytokines (e.g. IL-8) from airway epithelial cells *in vitro*.
- To identity any carbonaceous material in placental tissues from healthy full term pregnancies – as a proxy organ for particle translocation.
- To improve participants' knowledge on air pollution and provide advice on how to reduce personal pollution exposure.

Chapter 2: Methods

2. Methods

2.1. Ethical approval

The project protocol were reviewed and approved by the Joint Research Management Office for Barts Health NHS Trust and Queen Mary University of London.

For the Cystic Fibrosis project, ethical approval to recruit children was granted by the NHS Research Ethics Committees (REC, REC references 17/EM/0023, IRAS project ID 215879) in January 2017, following a full ethical review. Health Research Authority (HRA) approval was obtained in February 2017. See Appendix 4 for the latest version of the study protocol.

Five subsequent amendments were approved for the following reasons:

I. To expand the healthy recruit population and allow for sputum induction to be performed outside of the hospital.
II. To change sponsor details; and expand participant groups with the addition of non-CF bronchiectasis patients.
III. To include microbiology experiments on macrophages from children with CF.
IV. To update the European Union *General Data Protection Regulation (GDPR);* and add in the use of systemic over-the-counter dose of Ibuprofen in order to investigate the possibility of reducing *in vivo* prostanoids production.
V. To expand healthy recruits population to pan-London.

See appendix 7 for all correspondences with the ethics committee.

2.2. Participants

Children with cystic fibrosis were recruited from the Royal London Children's Hospital, one of the largest tertiary CF centres in the UK. Age-matched healthy controls were recruited from siblings of paediatric patients at the Royal London Hospital, and also through the research team's media and public engagement work across London. Since the Royal London Hospital is a specialist referral centre for north/ northeast London and Essex, the home addresses of the CF cohort scattered across central and greater London. In order to map the healthy cohort accordingly, siblings of patients were targeted as healthy recruits. However, in view of air pollution variations in and outside of London, all recruits were limited to those residing within the M25.

2.2.1. Inclusion criteria

I. Children with cystic fibrosis aged 1-17, residing within the M25 in London.

II. Age and gender-matched healthy controls residing within the M25 in London.

2.2.2. Exclusion criteria

I. Current active smoker.

II. Receiving immunosuppressive drug therapy.

III. For children with CF who were not on regular nebulised hypertonic saline: drop in FEV_1 of >15% post-bronchodilator (exclusion criterion for sputum induction).

IV. For healthy controls: post-bronchodilator FEV_1 <80% (standard exclusion criterion for sputum induction in healthy individuals).

V. For participants involved in other research study, they were excluded if the other research study had any potential impact on sampling or results of this study.

2.3. Recruitment process

Potential participants were referred by a member of their clinical care team, the research team then reviewed and ensured they met the eligibility criteria. Potential participants were approached individually. Age appropriate information sheets were available for participants and their parents, these were divided into age groups 1-6 years, 7-11 years, 12-15 years, and 16 years and above (examples of these information sheets can be found in appendix 5). Informed consent and assent were obtained at least 24 hours after dissemination of information sheets (examples of consent and assent forms can be found in appendix 6). Participants and parents who took part in activities which were added to the study at a later stage were asked to sign the modified consent and assent forms. Children with CF were approached and recruited first, followed by recruitment of healthy controls – this allowed for age, gender, and home address matching of the control group to the CF group.

2.4. Air pollution questionnaire

Participants and their parents were asked to fill in a one-page questionnaire following informed consent. The questionnaire contained questions on the home environment – cigarette smoking exposure, type of cook stove, ventilation method during cooking, use of candles; travel information – mode and duration of daily transport; and outdoor activities.

2.5. Exposure to air pollution

2.5.1. Modelled exposure at home address

The London Air Quality Toolkit (LAQT) is an established emissions dispersion model developed by the Environmental Research Group at King's College London. The LAQT includes all London Atmospheric Emissions Inventory (LAEI) sources such as road transport, industrial processes, railways, shipping, aviation, and agriculture. It incorporates meteorology and urban topology, producing detailed spatially accurate air quality maps – allowing for fore- and back-casting of air quality across the capital. It can model annual means of NO_X, NO_2, PM_{10}, $PM_{2.5}$ and O_3 in Greater London, at a resolution of 20 m x 20 m.

Colleagues at King's College London estimated the mean PM and NO_2 concentrations based on participants' home postcodes, 12 months prior to the date when participants underwent first sputum induction.

2.5.2. Black carbon monitoring

Participants' real-time black carbon (BC) exposure was measured by a pocket-sized microAeth AE51 aethalometer (firmware 709, Aethlab, California, USA). An aethalometer contains a built-in pump, with flow control and data storage capacity. The sampled air is collected on a Teflon coated glass fibre filter situated within the device. The aethalometer measures the rate of change in transmitted light absorption during continuous BC deposition on the filter, based on the fact that black material absorbs light the strongest. It can provide measurement resolution of 0.001 $\mu g/m^3$ with precision of ± 0.1 $\mu g/m^3$. The device battery is able to run for up to 24

hours on a single charge. Aethalometer technology has been used worldwide to measure BC exposure for over 30 years. The AE51 was chosen for this study because of its reliability from previous pollution studies across the world, and its portability and ease of operation.

Participants were asked to carry an aethalometer for 2 typical school days, using an activity diary (appendix 12) to record their mode and time of commute, and time spent in- and out-door (figure 2.1). The aethalometer was programmed to provide a reading (ng/m^3) every 60 seconds, at a flow rate of 100 mL/min. Mean per minute BC exposure (ng/m^3) across the two days was determined.

Figure 2.1. A participant carrying a portable aethalometer in a small backpack. The tip of the sampling tube (red arrow) was clipped to his jacket collar, sampling the air he was breathing in. Photo used with patient and parental permission.

2.5.3. Nitrogen dioxide monitoring

Nitrogen dioxide (NO_2) analysis using diffusive samplers (IVL Swedish Environmental Research Institute Ltd, Gothenburg, Sweden) is based on molecular diffusion of the gas. The gas molecules diffuse and are quantitatively collected onto a filter, giving a concentration value integrated over time, after adjusting for temperature and estimated air flow. Turbulent diffusion is prevented by a mesh overlying the filter. Results were analysed at the IVL Swedish Environmental Research Institute, expressed as average concentrations during the sampling time periods.

2.5.3.1. Indoor NO_2 monitoring

Participants were given a NO_2 diffusion sampler to be placed at home for two weeks – they were instructed to place this in a room, other than the bedroom, that the participant stayed in most.

2.5.3.2. Personal NO_2 monitoring

Participants carried a NO_2 diffusion sampler on their person (e.g. on school jacket or school bag) for two weeks.

2.6. Alveolar macrophage *in vivo* function

2.6.1. Spirometry

Spirometry was performed using MicroMedical MicroLab Spirometer (Care Fusion, UK) with a data management system compliant with American Thoracic Society (ATS)/ European Respiratory Society (ERS) 2005 guidelines [172]. Flow volume loops were displayed for immediate quality control. Both unadjusted lung function and percentage predicted values adjusted for age, ethnicity, gender and height were measured.

Spirometry was only performed in children over the age of 7 years for practical reasons – their ability to understand instructions and coordinate with good technique was crucial to generating meaningful readings. A baseline lung function test was performed, followed by inhalation of 400 µg (4 puffs) salbutamol, a short-acting β2 agonist, by metered dose inhaler (MDI) via a Volumetic spacer (Allen and Hanbury, UK). "Post-bronchodilator" lung function was performed 15 min after. Forced expiratory volume in 1 sec (FEV_1) and forced vital capacity (FVC) were measured to ensure participants fulfil the inclusion criteria set out above. A final lung function test was repeated following sputum induction to ensure no side effect such as bronchospasm had occurred.

2.6.2. Sputum induction

Sputum induction was performed according to the ERS guidelines [173], on all recruited children over the age of 7 years for practical reasons stated above. Sputum, containing alveolar macrophages, was induced using approximately 4 mL

of 3.5-7% saline via a portable Multisonic Profi 80180 nebuliser (Schill, Germany), nebulised at approximately 0.6 mL/min. Hypertonic saline was used to thin secretions in the lower respiratory tract, especially in mucous consolidation seen in CF. It therefore aided the upward movement and clearance of respiratory secretions mediated by ciliated cells.

400 µg Salbutamol, given prior to nebulised hypertonic saline administration, was used to limit the potential side effect of bronchospasm, hence the need for lung function monitoring.

Multiple induced sputum samples (limited to 1 sample per day) were obtained per participant, at separate house visits, due to the limited number of alveolar macrophages expectorated from each induction session. Each sample often contained enough alveolar macrophages for only one of the experiments described below.

2.6.2.1. Children with cystic fibrosis

For children with CF, hypertonic saline forms part of their daily routine chest physiotherapy treatment (figure 2.2). Their usual concentration (7%) of saline was used. Sputum induction was performed at a time and place most convenient to the participant, such as after school at home, during their routine clinic appointment, or during the beginning of their elective hospital admissions – these were limited to admissions with no signs of pulmonary exacerbations, such as lung function decline or respiratory symptoms. The timing of sputum induction was synchronised with the children's routine chest physiotherapy session to limit inconvenience. Sputum samples were collected into sterile pots and transported at 4^{o}C to the laboratory within 4 hr.

Figure 2.2. A participant receiving nebulised hypertonic saline for sputum induction during a home visit. Photo used with patient and parental permission.

2.6.2.2. Healthy children

For healthy controls, sputum induction was done either at the Royal London Children's Hospital, or, more commonly, at an appropriate and safe place which was convenient for the participants and their family, such as home visits after school. The same sputum induction protocol applied but the hypertonic saline concentration used was 3.5%. Healthy individuals produce less viscous respiratory secretions compared to the CF group, therefore a lower concentration of hypertonic saline would usually suffice and could limit the potential of bronchospasm.

2.6.3. Sputum processing

Traditional sputum processing protocols [174] recommend sample processing within 4 hr of collection, in order to ensure cell viability. However, owing to the fact that the majority of samples were collected in the evening after school hours, followed by 6 or more hours of experiments, the protocol was adapted accordingly. Sputum samples were kept in 20 mL of Dulbecco's phosphate-buffered saline with 2% Foetal Bovine Serum (FBS) (DPBS/FBS, STEMCELL, Canada) at 4^{o}C overnight, until processing the next morning – all samples were fully processed within 24 hr of collection. Despite overnight storage, alveolar macrophage viability was maintained with preserved phagocytic ability seen *in vitro*.

Whole sputum was diluted 1:1 in Dulbecco's phosphate buffered saline (Corning, USA) and agitated, followed by centrifugation at 3500 rpm (2401 x g) for 10 min. The pellet was resuspended in 1 mL 0.1% Dithiothreitol (DTT) (Fisher Scientific, USA), agitated by vortex and gently rocked at room temperature for 15 min. The suspension was then passed through a 36 µm gauze (NITEX 03-

36/28, Sefar, Switzerland) and centrifuged at 10000 rpm (7840 x g) for 10 min. The pellet was resuspended in 1 mL DPBS/FBS (2%). An aliquot of 35 µL was transferred and secured in a Shandon™ single Cytofunnel™ (ThermoFisher Scientific, UK) and spun at 1500 rpm (277 x g) for 3 min, followed by staining with Hemacolor® (Merck, Darmstadt, Germany). The glass slide was air dried and mounted with a glass coverslip using Vectamount glue. The cytospin slides were used to analyse alveolar macrophage black carbon loading. The remaining samples underwent macrophage enrichment as described below (figure 2.3).

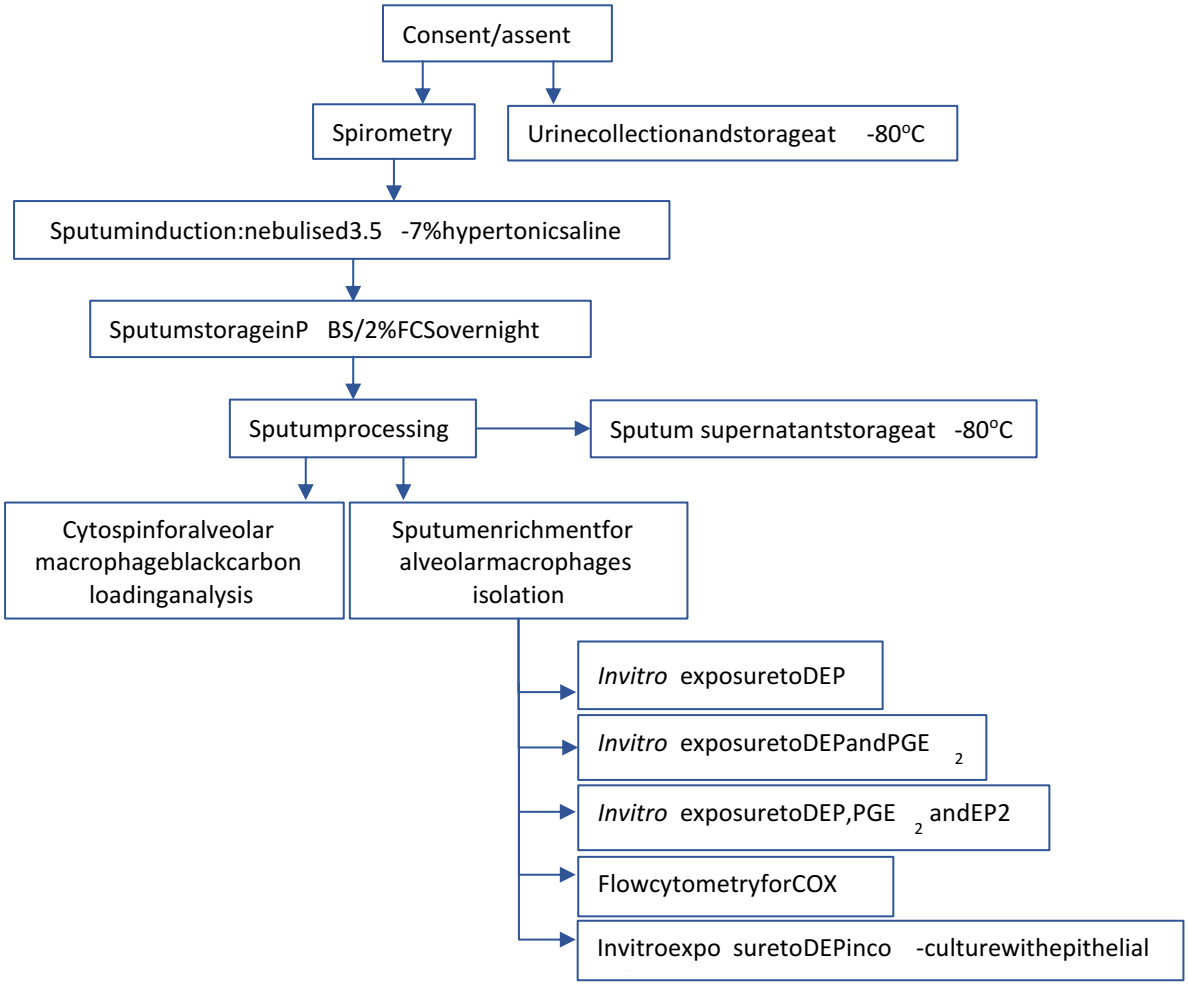

Figure 2.3. Flow diagram showing sequence of sputum processing for various experiments – further details of each experiment are discussed later in this chapter.

2.6.3.1. Protocol adaptation for cystic fibrosis

Traditionally, sputum was processed by selecting cell-rich plugs of airway mucus before processing. However, this was modified due to the presence of viscous mucus in CF, rendering plugs picking challenging, with abundant cell loss. Whole sputum samples were therefore used and diluted with 20-40 mL of DPBS depending on sputum consistency, followed by centrifugation and pellet resuspension as described above.

2.6.4. Alveolar macrophage black carbon analysis

Alveolar macrophage black carbon (AMBC) was analysed as previously described [101]. Briefly, light microscopy was used to visualise AMs. Digital colour images of 50 randomly selected AMs from each participant were captured using a stereo-histology microscope (Mazurek Optical Services, UK) at x100 objective under immersion oil using PictureFrame 2.2 computer software. The images were imported into ImageJ 1.50i (National Institute of Health, USA).

Each AM was outlined manually using a freehand tool (figure 2.4a). The software measured the number of pixels within the outlined area, and converted that into a dimension (μm^2) – i.e. size of the AM. The software was set to correspond 1473 pixels to 100 μm^2 when imaging at x100 objective.

Areas of inorganic carbon (stained black) deposited within each AM were distinguished from other darkly stained substances such as the nucleus and adherent or phagocytosed bacteria (stained dark purple). The black (carbonaceous particulates) areas were selected with the freehand tool (shown in yellow, figure

2.4b), the image was then converted to a 32-bit greyscale image (figure 2.4c), followed by adjustment of the image threshold in order to identify the most darkly coloured areas (shown in red, figure 2.4d). The software measured the red area within the pre-selected yellow boundary – this was the AMBC load. The mean AMBC (μm^2) of 50 AMs from each participant was determined.

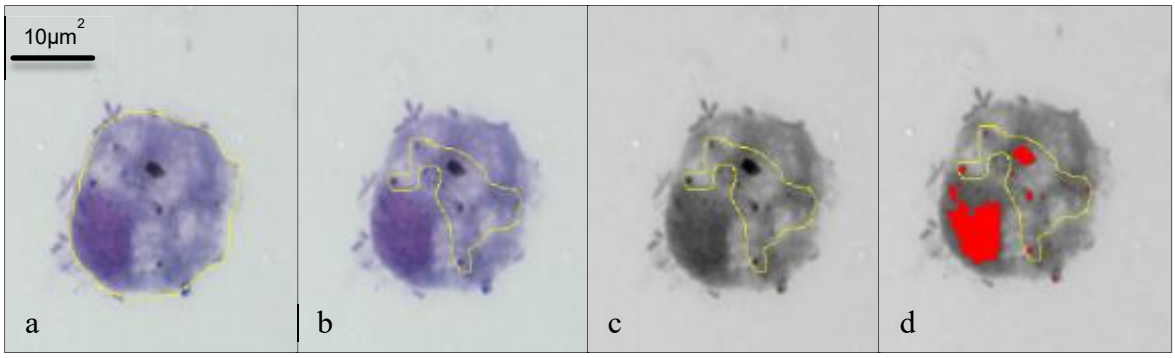

Figure 2.4. Measuring alveolar macrophage black carbon: (a) Area of an alveolar macrophage outlined (in yellow) by a freehand tool and the area (μm^2) is measured. (b) Areas of black carbon outlined by freehand tool. (c) Image converted to 32-bit greyscale. (d) Image threshold adjusted to highlight the darkest areas (shown in red).

A second observer blinded to the participants' health status (CF v control) repeated the image analysis for 11 randomly selected samples; agreement between both observers was determined using the Bland-Altman method.

2.7. Alveolar macrophage *in vitro* function

2.7.1. Sputum purification and alveolar macrophage Isolation

Following the cytospin slide production, the remaining suspension for each sputum sample was enriched to isolate alveolar macrophages. 40 µL of cell depleted erythrocytes and 60 µL of human monocyte enrichment cocktail (RosettSep, STEMCELL, Canada) were added to the suspension, followed by 20 min rocking at room temperature. Concentrations were modified from the manufacturer's protocol (30 µL of cell depleted erythrocytes and 50 µL of human monocyte enrichment cocktail); the modified concentrations provided a better purification yield with >98% of enriched cells being macrophages.

Cell depleted erythrocytes were obtained from fresh human capillary blood, drawn on the day of sputum processing. 1 mL of fresh human blood was diluted with 0.6 ml incomplete (supplemented with 4% FBS) Roswell Park Memorial Institute (RPMI-1640) medium (Gibco, Thermo Fisher Scientific, USA) before suspension on an equal volume of density gradient medium, Ficoll-Paque Plus (GE Healthcare, Sweden), and centrifugation at 1821 rpm (650 x g) for 22 min. RPMI dilution was conducted to reduce blood viscosity and improve the separation process. Cell depleted erythrocytes were collected as a pellet. Other sources of erythrocytes including refrigerated horse blood, refrigerated human blood and frozen human erythrocytes, were trialled but had suboptimal yield of AM enrichment – refrigerated horse and human blood did not separate properly in density gradient medium for erythrocytes isolation; frozen human erythrocytes resulted in poor separation in the monocyte enrichment step.

The cell suspension with erythrocytes and enrichment cocktail was diluted 1:1 in PBS/FBS (2%), suspended on density gradient medium (1:1), and centrifuged at 2020 rpm (800 x g) for 30 min. The resulting monolayer of enriched AMs was aspirated using a 3 ml Pasteur pipette (Sarstedt, Germany) from the density medium: plasma interface. The enriched cells were washed in 500 µL of PBS/FBS (2%) to remove residual Ficoll-Paque. The final cell pellet was resuspended in appropriate cell culture medium for investigations.

Enrichment of AMs enabled functional assessment of AMs alone, without the presence of mucus, inflammatory cells, epithelial cells, and bacteria, thus allowing clear visualisation of AMs and their contents. This was particularly useful in CF where the sputum samples were often heavily laden by these materials (figure 2.5).

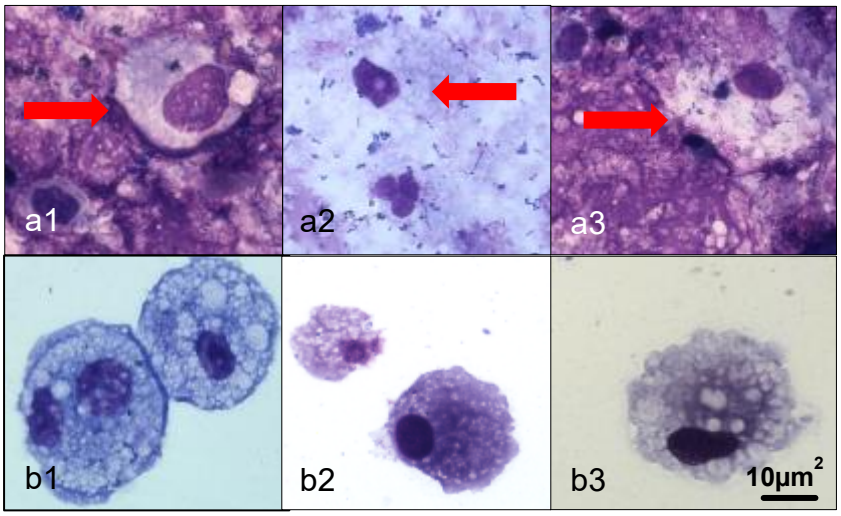

Figure 2.5. (a) Cytospin slides from children with CF, heavily laden with mucus and bacteria, making visualisation of cells and image analysis very difficult; alveolar macrophages identified with red arrows. (b) Enriched alveolar macrophages from children with CF, with the majority of mucus, bacteria, and other cells (e.g. epithelial cells, neutrophils, etc) removed.

2.7.2. Alveolar macrophage uptake of diesel exhaust particles

Nunc™Lab-Tek™ cell culture chambers (ThermoFisher Scientific, UK) were pre-coated in 1:5 collagen (calf skin, Sigma-Aldrich, Missouri, USA) for at least 30 min at 37 °C. 1:10 collagen concentration was tested, but due to limited number of AMs available from each sample, cell adhesion was encouraged to avoid cell loss, hence the increase in concentration.

Enriched AMs were suspended in chamber wells and allowed to adhere overnight at 37°C, 5% CO_2 in complete RPMI supplemented 10% FBS and Penicillin-Streptomycin (Sigma-Aldrich, Missouri, USA).

Cells were washed the following day with incomplete RPMI. Experimental chamber wells were exposed to 10 µg/mL of diesel exhaust particles (DEP) (SRM 2975, NIST, USA) for 2 h at 37°C, 5% CO_2; control wells were unexposed. Cells were subsequently washed with incomplete RPMI, chambers were removed and slides washed before staining (Hemacolor®, Merck, Darmstadt, Germany) and imaging by light microscopy using a stereo-histology microscope (Mazurek Optical Services, UK).

AM phagocytosis of DEP was assessed by image analysis as described above. As the number of AMs extracted varied between individuals, image analysis was kept consistent by using the same number of cells, ranging from 10 to 50 cells per participant, pre- and post- DEP exposure.

2.7.2.1. Choice of media and antibiotics

RPMI is a widely used basal medium for mammalian cell culture. Standard complete RPMI is supplemented with FBS and penicillin-streptomycin. However, sputum samples from CF participants were often laden with bacteria due to their disease process and organism colonisations. As a result, some CF samples had substantial bacterial growth during the overnight incubation period for cell adhesion – bacterial growth affected subsequent AM phagocytosis of DEP and impeded visualisation of AMs under light microscopy (figure 2.6). It was therefore decided that complete RPMI should also be supplemented with Primocin™ (Invivogen, Tolouse, France). Penicillin-streptomycin is effective against gram positive and gram negative bacteria; while primocin is effective against not only gram positive and negative bacteria, but also mycoplasma and fungi, and is non-toxic to primary cells. However, it was noted that some cells did not withstand primocin well, resulting in poor cell survival and adhesion overnight. Thus, for each sputum sample, standard complete RPMI (with FBS and penicillin-streptomycin) was used in half the chamber wells, and RPMI with primocin was used in the other half.

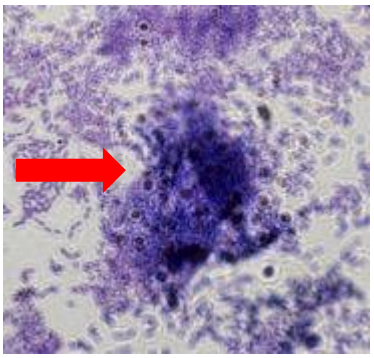

Figure 2.6. Enriched alveolar macrophage (red arrow) from a participant with CF. Extensive bacterial growth overnight, impeding visualisation of AM.

2.7.2.2. Concentration of diesel exhaust particles

The DEP concentration of 10 µg/mL was informed by earlier research [102]. Higher concentrations (20 and 40 µg/mL of DEP) were tested and a dose-dependent response was seen (figure 2.7). Concentrations above 10 µg/mL was proved to render visualisation of AM intracellular content difficult (figure 2.8), therefore 10 µg/ml was used despite it not being a maximum concentration.

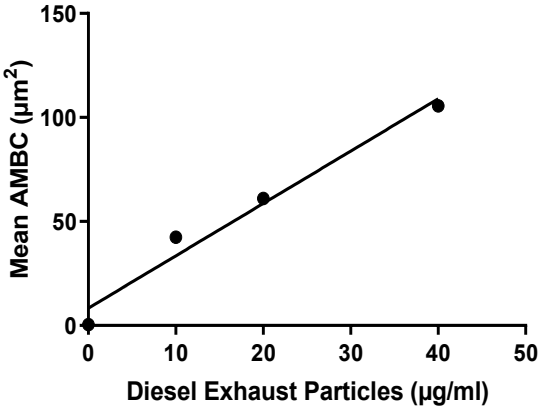

Figure 2.7. Dose-dependent response in alveolar macrophage phagocytosis (mean AMBC of 50 randomly selected AMs) after 2 h exposure to 0, 10, 20 and 40 µg/mL of diesel exhaust particles. Alveolar macrophages isolated from a healthy individual.

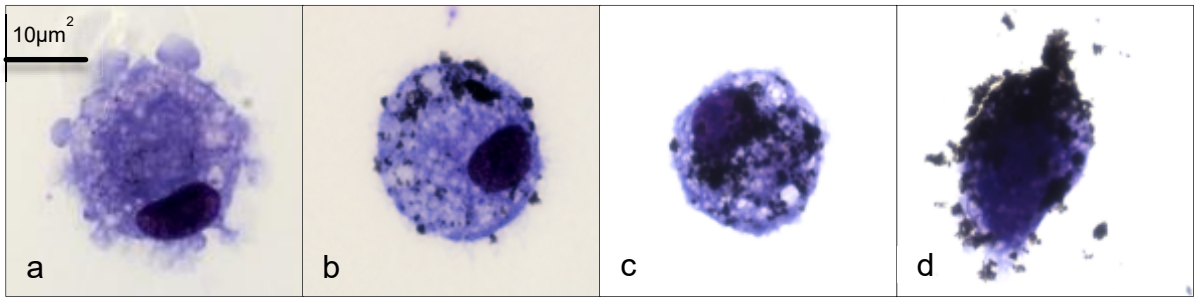

Figure 2.8. Alveolar macrophages isolated from a healthy individual, showing dose-dependent responses to diesel exhaust particles (DEP) exposure: 0, 10, 20, and 40 µg/mL of DEP in (a), (b), (c) and (d) respectively. 10 µg/mL (b) was the final chosen concentration as intracellular contents were difficult to analyse accurately with higher concentrations of DEP (c and d).

One limitation of image analysis using light microscopy is the difficulty in distinguishing black carbon phagocytosed from those adhering to the cell surface, however, the distribution of black carbon suggests that the carbon load has been phagocytosed.

2.7.3. Effects of prostaglandin on alveolar macrophages function

Using enriched AMs isolated from both CF and control groups, the effect of prostaglandin-E2 (PGE_2) on AM phagocytosis was assessed by adding synthetic PGE_2 (P0409, Sigma-Aldrich, USA) to the cell-DEP culture, followed by image analysis. Briefly, following AMs enrichment, cells were allowed to adhere in chamber wells overnight as previously described, the chamber wells washed and resuspended in incomplete RPMI, and divided into two groups: i) 10 µg/mL of DEP; and ii) 10 µg/mL of DEP and 10^{-6} M of PGE_2. PGE_2 was added to the group (ii) chamber wells 15 min before all wells were exposed to 10 µg/mL of DEP for 2 h. The concentration of PGE_2 was informed by the literature [102,141]. As before, the entire population of AMs extracted from each sample was equally divided between the two cultures. Image analysis was kept consistent by using the same number of cells, ranging from 10 to 50 AMs per participant, between cultures with and without PGE_2. Using light microscopy, mean AMBC of 10 to 50 cells with and without the presence of PGE_2 were compared.

2.7.4. Effects of EP2-receptor antagonist on alveolar macrophages function

To further confirm the relationship between PGE_2 and phagocytosis, the effect of PGE_2 was reversed by the addition of an EP2-receptor antagonist (AH6089, Sigma-Aldrich, USA) to the cell-DEP culture.

Responder AMs from healthy participants were used for this analysis. Enriched AMs were allowed to adhere in chamber wells overnight. These were divided into three groups; i) 10 µg/mL of DEP; ii) 10 µg/mL of DEP and 10^{-6} M of PGE_2; and iii) 10 µg/mL of DEP, 10^{-6} M of PGE_2 and 75µM of EP2-receptor antagonist [175]. The EP2-receptor antagonist was added to group (iii) wells and left for 1 h at 37°C, followed by addition of PGE_2 to groups (ii) and (iii) for another 15 min, before all wells were exposed to DEP for 2 h. Image analysis was performed as previously described, using the entire population of extracted AMs from each participant, while keeping the number of AMs in each culture consistent.

2.7.5. Expression of cyclooxygenase 2 in alveolar macrophages

Enriched AMs from both groups of participants were washed and resuspended in PBS/FBS (2%) before being fixed, permeabilised and labelled with antibodies for flow cytometry.

2.7.5.1. Fixation and permeabilisation

Using a cell fixation and cell permeabilisation kit (FIX & PERM, Thermo Fisher Scientific, USA), enriched AMs were fixed and permeabilised. The kit allowed for

preservation of the morphological scatter characteristics of the cells, enabling intracellular detection of enzymes (e.g. cyclooxygenase 2). Fixing reagent was added to the cells for 15 min and washed in PBS/FBS (2%), centrifuged at 1200 rpm (218 x g) for 3 min, the supernatant discarded, followed by pellet resuspension in permeabilisation reagent and left for 20 min at room temperature.

2.7.5.2. Antibody labelling and macrophage marking

At the same time as exposure to the permeabilisation reagent, cells were labelled with anti-CD11b macrophage markers (1:100, Abcam, ICRF44), and COX-2 primary antibodies (1:200, Abcam, UK, EP1978Y), followed by washing as above, and labelling with secondary antibodies (1:2000, Abcam, UK) in the dark for 20 min at room temperature (figure 2.10). The secondary antibodies for COX-2 conjugated to Alexa Fluor 488 (Goat anti-Rabbit IgG H&L, A42731), and the macrophage marker secondary antibodies conjugated to Alexa Fluor 647 (Goat anti-mouse IgG H&L, A32728). Cells were washed again before resuspension in DPBS for flow cytometry. An isotype control was used to adjust for non-specific immunostaining.

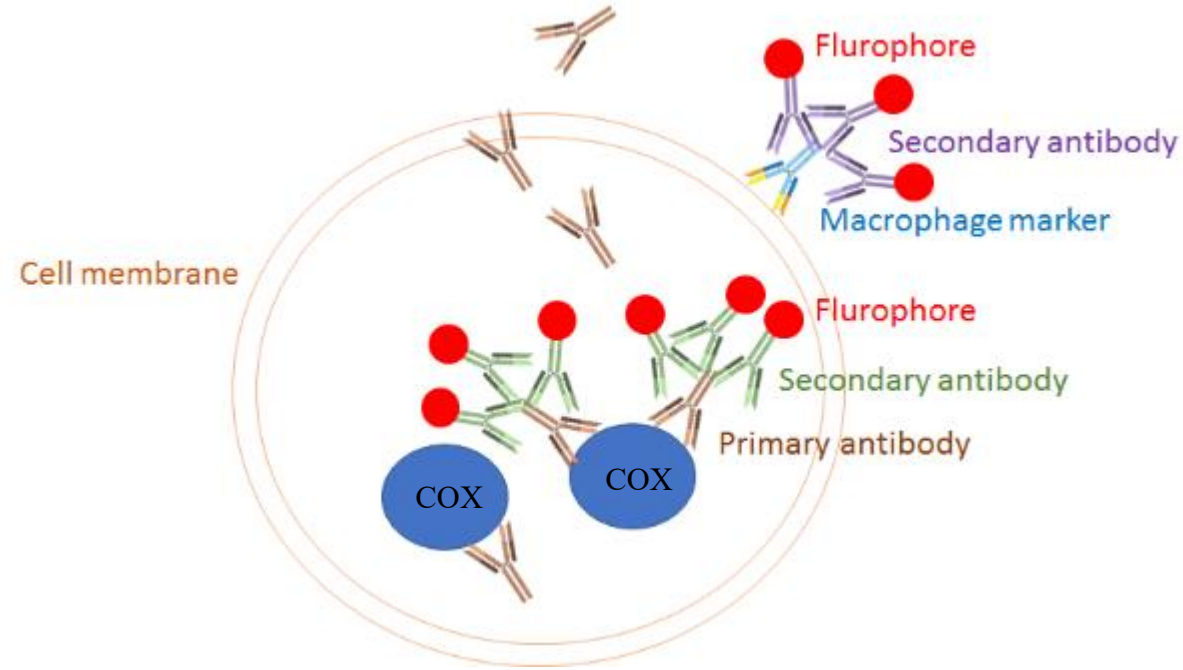

Figure 2.10. Cell fixation and permeabilisation, allowing antibodies to penetrate following membrane permeabilisation. COX was labelled with primary antibodies, which were then labelled with secondary antibodies conjugated to fluorophore. The cell (macrophage) was also labelled with macrophage markers which were labelled with secondary antibodies conjugated to fluorophore as well.

2.7.5.3. Flow cytometry

Data was acquired using the BD FACS Canto II flow cytometer and BD FACSDiva software version 6.1.13 (BD Biosciences, UK) to determine the median fluorescence intensities (MFI) for COX-2 expression in AMs from both CF and control groups.

2.7.5.4. Flow cytometry analysis

CD11b is traditionally used as a marker for myeloid cell types. It has been shown that CD11b is expressed on all human alveolar macrophages, although the extent is variable between individuals [176]. The cell populations used for flow cytometry had

been enriched by monocyte enrichment cocktail to isolate AMs, the addition of macrophage markers during antibodies staining further enhanced the AM isolation.

2.7.5.5. Gating strategy

Using forward and side scatter, the larger and more granular population was selected (figure 2.11a, population P1). The APC fluorophore channel allowed for identification of $CD11b^{high}$ cells (figure 2.11b,c, population P6). Using the FITC fluorophore channel and eliminating non-specific isotype staining, the final cell population was identified (figure 2.11d, e), and MFI determined.

Due to the small number of cells available per participant, slight variation in gating strategy between different observers could result in drastic changes in MFI values. Therefore, the same observer repeated the analysis on a randomly selected set of data, while blinded to the participants' health status (CF vs control). The agreement between blinded and unblinded analyses was determined using the Bland-Altman method.

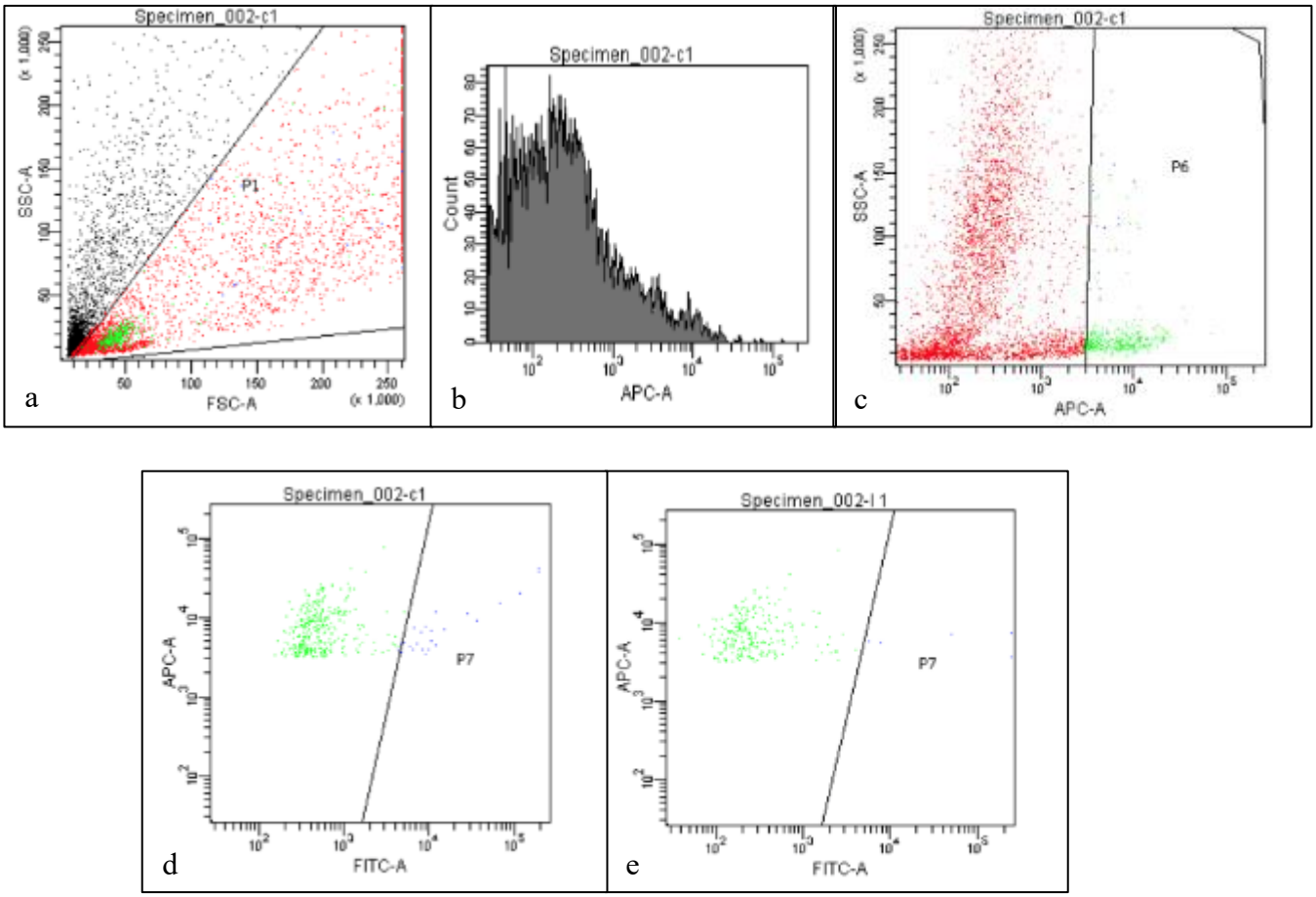

Figure 2.11. Flow cytometry gating strategy. (a) Large and granular population selected (P1). (b) and (c) APC-A channel to help gate CD11b^{high} cell population (P6). (d) Gating of final cell population (P7) while eliminating non-specific isotype staining (e).

2.8. Prostanoid profiles in cystic fibrosis vs healthy controls

2.8.1. Urinary analysis for prostanoids

Due to the chemical instability of prostaglandins (PGs), it is often difficult to directly measure them in bodily fluids. It is therefore generally accepted to measure urinary metabolites of prostanoids as a reflection of endogenous PG synthesis [177].

Urine samples were collected from both CF and control groups immediately after first sputum induction – for the CF group, samples were collected during clinically stable state. Samples were transported to the laboratory on ice, followed by storage at -80^{o}C within 1 h, until analysis by high performance liquid chromatography–tandem mass spectrometry (HPLC-MS). This was performed at the Jagiellonian University Medical College, Krakow, Poland by Professor Marek Sanak's team, who provided the following methodology.

Creatinine concentration in urine was measured using a small volume of 0.15 mL per sample on COBAS Integra 400 plus analyser with a clinically validated assay. 0.5 mL of urine sample was added to deuterated internal standard mixture (2ng 13,14-dehydro-15-keto-PGE_2-d4, 13,14-dehydro-15-keto-PGD_2-d4, 8-iso-$PGF_{2\alpha}$-d4, 9α,11β-PGF_2-d4, 10ng tetranor-PGE-M-d6, tetranor-PGD-M-d6 and 2ng LTE_4-d3). After acidification with acetic acid, samples were extracted to the organic phase using a mixture of 80% tert-buthyl-ether and 20% methanol. The solvent was next evaporated and the residue was reconstituted in 40 µl methanol.

Quantitation of eicosanoids was performed using HP-LC with autosampler for analysis (Prominance UHLC, Shimadzu Corporation, Kyoto, Japan), on Kinetex Biphenyl column 100 mm x 2.1 mm 2.5 µm (Phenomenex), in negative ionization.

Details on analytes measured, which included additionally prostaglandins systemic metabolites are presented below.

Compound	MRM [m/z]	CE [eV]	Confirmatory MRM [m/z]	CE [eV]	Rt [min]
Tetranor-PGE-M-d0	327>143	-30	327>309	-20	11.88
Tetranor-PGE-M-d6	333>149	-32	333>315	-20	11.83
Tetranor-PGD-M-d0	337>143	-30	327>309	-20	12.12
Tetranor-PGD-M-d6	333>149	-32	333>315	-20	12.07
13,14-dehydro-15-keto-PGE2-d0	351>333	-16	351>175	-24	16.12
13,14-dehydro-15-keto-PGE2-d0	355>337	-16	355>179	-26	16.09
13,14-dehydro-15-keto-PGD2-d0	351>175	-24	351>333	-16	16.51
13,14-dehydro-15-keto-PGD2-d0	355>179	-26	355>337	-16	16.47
LTE4-d0	438>333	-27	440>289	-30	17.92
LTE4-d3	441>336	-27	443>291	-30	17.89

Quantitation of urinary prostaglandins: 8-iso-$PGF_{2\alpha}$, 9α,11β-PG_{F2} and thromboxanes: TXB_2 and 11-dehydro-TXB_2 was performed using gas chromatography (GC) by evaporation and derivatization. These were sequential chemical reactions to render the analytes necessary volatility by modifications of carboxyl group (15 µl 10 % pentafluorobenzyl bromide (PFBBr) in acetonitrile with 10% N-ethyldiisopropylamine (DIPE) in acetonitrile). After formation of pentafluorobenzyl ester, samples was evaporated and reconstituted in 30 µl methanol and next cleaned up using thin-layer chromatography (TLC) using a mobile phase of ethyl :n-heptane (90:10). After collection of silica gel with the analyte at 7-19 mm for 8-iso-$PGF_{2\alpha}$, 9α,11β-PGF2, 38-50 mm for TXB_2 and 61-69

mm for 11dehydro TXB_2 with 1 mL of ethyl acetate and evaporation, hydroxyl groups were blocked with BSTFA (Bis(trimethylsilyl)trifluoroacetamide) in pyridine. Next, evaporated sample was dissolved in toluene (50 µl). GC-MS/MS analysis was done using on Shimadzu GC-2010 plus (Kyoto, Japan) instrument in a single ion monitoring (SIM) mode and capillary column Zebron 5MS (30 m x 0.25 mm x 0.25 µm, Phenomenex).

Described methods were validated for linearity using calibration curves. All analytes reported were above the lowest limit of detection. Concentration of each eicosanoid was calculated from the area under the peak on MRM or SIM trace, using internal deuterated standard peak as a reference (denominator). This method compensates for recovery errors during preparation and is referenced as a stable isotope dilution quantification. Results were reported in picograms per milligram of creatinine.

All the solvents were of HPLC grade and purchased from Mallincrodt Baker, Inc. (Phillipsburg, NJ, USA), while other chemicals were from Sigma–Aldrich Co. (St. Louis, MO, USA).

Eicosanoid metabolites measured are listed in Table 2.2, along with their eicosanoid precursors.

Urinary eicosanoids metabolites	Eicosanoid precursors
13,14-dihydro-15-keto-E2	15-oxo-prostaglandin E2
13,14-dihydro-15-keto-D2	Prostaglandin D2
13,14-dihydro-15-keto-tetranor-E2	Prostaglandin E2
13,14-dihydro-15-keto-tetranor-D2	Prostaglandin D2
Tetranor PGEM *	Prostaglandin E2
Tetranor PGDM *	Prostaglandin D2
15-doexy-delta12,14-PGJ2	Prostaglandin J2
9a,11b-PGF2	Prostaglandin D2

Table 2.1. Panel of urinary prostanoid metabolites analysed, with their prostanoid precursors. * Major urinary metabolites of the specified PG.

2.8.2. Sputum supernatant analysis for prostanoids

For measurement of PGs and their metabolites in sputum supernatant, a different approach to sputum processing [178] was employed in order to maintain equal dilution factor for both CF and control groups. The previously described method involved diluting the sputum samples with various amount of DPBS, depending on the sputum viscosity. Variable dilutions, volumes and weights made adjusting PG metabolites levels challenging.

Briefly, whole sputum sample was weighed before adding DTT (1:1), followed by shaking on a rocker for 15 min at 37°C. The mixture was then filtered through a 36 µm gauze, followed by weighing and centrifugation of the filtrate at 10000 rpm (7840 x g) for 10 min. Cell-free supernatant of sputum samples after gauze filtration was stored at -80°C until analysis by HPLC-MS.

Protein concentration was measured in 0.15 mL of each sample on COBAS Integra 400 plus analyzer using a clinically validated assay. Total sputum supernatant sample (volume from 1.2 to 28 mL) was thawed on ice and next spiked in with a mixture of 2 ng each of deuterated, chemically identical standards (5-HETE-d8, 12-HETE-d8, 15-HETE-d8, PGE_2-d4, 8-iso-PGE_2-d4, PGD_2-d4, PGA_2-d4, 13,14-dehydro-15-keto-PGE_2-d4, 13,14-dehydro-15-keto-PGD_2-d4, 8-iso-PGF_α-d4, 9α,11β-PGF_2-d4, TXB_2-d4, 11-dehydro-TXB_2-d4, tetranor-PGE-M-d6, tetranor-PGD-M-d6 and 4ng LTE_4-d3, LTD_4-d5, LTB_4-d4, 5-oxo-ETE-d7). The samples were adjusted to pH 4.5 with acetic acid and were centrifuged at 2500 x g for 10 min at 4ºC to remove precipitates. A solid phase extraction (SPE) was done using JT Baker SPE-12 g Extraction System (JT Baker Chemical Company Netherlands). Each

sample was passed through an individual cartridge (Waters C-18) preconditioned with methanol (5 mL) and distilled water (5 mL). Following binding of eicosanoids, two washes of the cartridge were done with distilled water (5 mL) and n-heptane (5 mL) to remove impurities. Eicosanoids were eluted form the cartridge with methanol (5 mL). Next, methanol solution was evaporated under nitrogen and the residue was reconstituted in 50 µl methanol. HPLC column injection was 5 µl of the sputum supernatant extract, HPLC with autosampler was used for analysis (Prominance UHLC, Shimadzu Corporation, Kyoto, Japan). Conditions for chromatographic separation were as follows:

- HPLC Column Zorbax Eclipse XDB-C18 50 mm x 1mm 3.5 µm (Agilent)
- Column Kinetex Biphenyl 100 mm x 2.1 mm 2.5 µm (Phenomenex) – two different gradients of elution were used (see below).

Eicosanoids were quantified using multiple reaction monitoring mode (MRM) tandem mass spectrometry (Qtrap 4000, Applied Biosystems, Foster City, CA, USA) equipped with electrospray ion source, in four separate runs including positive and negative athmospheric pressure ionization. Details of individual compound analysis were:

Positive mode (Zorbax Eclipse)

Compound	MRM [m/*z*]	CE [eV]	Confirmatory MRM [m/*z*]	CE [eV]	Rt [min]
LTE_4-d0	440>189	25	440>301	15	7.30
LTE_4-d3	443>192	22	443>304	15	7.27
LTD_4-d0	497>189	25	497>301	18	7.38
LTD_4-d5	502>194	25	502>306	18	7.35

Negative mode (Zorbax Eclipse)

Compound	MRM [m/*z*]	CE [eV]	Confirmatory MRM [m/*z*]	CE [eV]	Rt [min]
LTB_4-d0	335>151	-35	335>129	-38	8.16
LTB_4-d4	339>153	-35	339>130	-38	8.13
5-HETE-d0	319>115	-20	319>257	-18	9.93
5-HETE-d8	327>116	-22	327>265	-18	9.89
12-HETE-d0	319>179	-21	319>257	-18	9.74
12-HETE-d8	327>184	-21	327>265	-18	9.70
15-HETE-d0	319>219	-19	319>257	-18	9.51
15-HETE-d8	327>226	-19	327>265	-18	9.49
5-oxo-ETE-d0	317>203	-30	317>163	-25	10.10
5-oxo-ETE-d7	324>210	-25	324>167	-25	10.06
11-dehydro-TXB_2-d0	367>161	-24	367>305	-16	6.16
11-dehydro-TXB_2-d4	371>165	-26	371>309	-16	6.12

Mobile phase: A 80% H_2O+20% ACN + 0.01% CH_3COOH

B 55% ACN+45% IPA + 0.01% CH_3COOH

Flow rate: 0.11 µl/min, column temperature 40ºC.

Gradient was used as follows: 0-1 min 8%B, 1-8 min 95% B, 8-10 min 95%, 10-12 min 100% B, 12-14 min 100% B 14-18 min 8% B – equilibrate 6 min.

Negative mode (Kinetex Biphenyl)

Compounds	MRM [m/z]	CE [eV]	Confirmatory MRM [m/z]	CE [eV]	Rt [min]
8-iso-PGE2-d0	351>271	-24	351>189	-38	36.90
8-iso-PGE2-d4	351>275	-26	339>193	-34	36.83
PGE2-d0	351>271	-24	351>189	-38	37.82
PGE2-d4	351>275	-26	339>193	-34	37.75
PGD2-d0	351>271	-24	351>189	-38	38.49
PGD2-d4	351>275	-26	339>193	-34	38.39
PGA2-d4	333>271	-24	333>189	-30	44.42
PGA2-d4	337>274	-26	337>193	-30	44.32
13,14-dehydro-15-keto-PGE2-d0	351>333	-16	351>175	-24	41.92
13,14-dehydro-15-keto-PGE2-d4	355>337	-16	355>179	-26	41.81
13,14-dehydro-15-keto-PGD2-d0	351>175	-24	351>333	-16	43.99
13,14-dehydro-15-keto-PGD2-d4	355>179	-26	355>337	-16	43.87

Mobile phase: A 100% H2O + 0.01% CH_3COOH

B 55% ACN+45% IPA + 0.01% CH_3COOH

Flow: 0.1 µl/min, column temperature 40ºC

Gradient 0-1 min 12%B, 1-50 min 45% B, 50-60 min 100%, 60-68 min 100% B, 68-78 min 12% B – equilibrate 6min.

Negative mode (Kinetex Biphenyl)

Compound	MRM [m/z]	CE [eV]	Confirmatory MRM [m/z]	CE [eV]	Rt [min]
Tetranor-PGE-M-d0	327>143	-30	327>309	-20	11.88
Tetranor-PGE-M-d6	333>149	-32	333>315	-20	11.83
Tetranor-PGD-M-d0	337>143	-30	327>309	-20	12.12
Tetranor-PGD-M-d6	333>149	-32	333>315	-20	12.07

Mobile phase: A 100% H_2O + 0.01% CH_3COOH

B 100% ACN + 0.01% CH_3COOH

Flow: 0.11 µl/min, column temperature 40ºC

Gradient 0-1 min 0%B, 1-20 min 95% B, 20-22 min 100%, 22-25 min 100% B, 25-30 min 0% B – equilibrate 6min.

After HPLC analysis, methanol extract of eicosanoids was prepared for gas chromatography (GC) by evaporation and derivatization. These were sequential chemical reactions to render the analytes necessary volatility by modifications of carboxyl group (15 µl 10 % pentafluorobenzyl bromide (PFBBr) in acetonitrile with 10% N-ethyldiisopropylamine (DIPE) in acetonitrile). After formation of pentafluorobenzyl ester, samples was evaporated and reconstituted in 30 µl methanol and next cleaned up using thin-layer chromatography (TLC) using a mobile phase of ethyl :n-heptane (90:10). After collection of silica gel with the analyte at 7-19 mm for 8-iso-$PGF_{2\alpha}$, 9α,11β-PGF2, 38-50 mm for TXB_2 and 61-69 mm for 11dehydro TXB_2 with 1mL of ethyl acetate and evaporation, hydroxyl groups were blocked with BSTFA (Bis(trimethylsilyl)trifluoroacetamide) in pyridine. Next, evaporated sample was dissolved in toluene (50 µl). GC-MS/MS analysis was done using on Shimadzu GC-2010 plus (Kyoto, Japan) instrument in a single ion

monitoring (SIM) mode and capillary column Zebron 5MS (30 m x 0.25 mm x 0.25 µm, Phenomenex).

Compound	SIM [m/z]	Rt [min]
8-iso-$PGF_{2\alpha}$-d0	569	12.52
8-iso-$PGF_{2\alpha}$-d4	573	12.49
9α,11β-PGF_2-d0	569	12.78
9α,11β-PGF_2-d4	573	12.75
TXB_2-d0	585	13.49
TXB_2-d4	589	13.45
11-dehydro-TXB_2-d0	511	16.08
11-dehydro-TXB_2-d4	515	16.04

Results were reported in picograms per milligram of protein.

2.8.3. Effects of CF supernatant on responder alveolar macrophage

To test the hypothesis that phagocytosis-inhibitory factors (e.g. PGE_2) are found within the CF airways, supernatant from CF sputum was added to enriched responder AMs isolated from healthy participants. All sputum samples from the CF group were processed as described above, following dilution with DPBS and centrifugation – prior to addition of DTT – supernatant was collected and stored at -80^{o}C until experiment. Responder AMs were isolated and allowed to adhere in chamber wells overnight as above. After washing and resuspending chamber wells with incomplete RPMI to a final volume of 300 µL, wells were divided into two groups: i) 10 µg/mL of DEP; ii) 10 µg/mL of DEP and 150 µL of CF supernatant. The CF supernatant was added simultaneously with incomplete RPMI, cells were exposed to DEP for 2 h. CF supernatant from different patients were used for experimental replicates with responder AMs from different healthy participants. CF supernatant was selected at random each time. The number of AMs used for

subsequent image analysis was kept consistent (ranged from 10 to 50 cells) between cultures.

2.8.4. Effects of EP2-receptor antagonist on responder alveolar macrophages cultured in CF supernatant

Since PGE_2 was the suspected inhibitory factor present in CF airways, an EP2 antagonist was used antagonise its effects in CF supernatant. CF supernatant and responder AMs were prepared as described before. Adhered AMs were washed and chamber wells resuspended with incomplete RPMI to a final volume of 300 µL, these were divided into three groups: i) 10 µg/mL of DEP; ii) 10 µg/mL of DEP and 150 µL of CF supernatant; and iii) 10 µg/mL of DEP, 150 µL of CF supernatant and 75µM of EP2-receptor antagonist. EP2-receptor antagonist was added to group (iii) for 1 h, followed by the addition of CF supernatant to groups (ii) and (iii), before exposing all wells to DEP for 2 h. Consistent number of AMs (ranged from 10 to 50 cells) between cultures were used for image analysis.

2.8.5. Effects of cyclooxygenase 2 inhibitors on prostaglandin production

Ibuprofen, a non-steroidal anti-inflammatory drug (NSAID) which works by inhibiting the activities of COX (both COX-1 and COX-2), was used to reduce the production of prostaglandin in children with CF. Urine samples were collected from the children before and after a 3-day course of Ibuprofen (dosage according to the British National Formulary, three times a day). Urinary metabolites of prostaglandin were

measured by high performance liquid chromatography–tandem mass spectrometry as described before.

2.9. Modelling the effects of impaired alveolar macrophages phagocytosis *in vitro*

2.9.1. The *in vitro* epithelium-macrophage model

To investigate the potential consequences of non-phagocytosed inhaled carbonaceous particles in the airways, an *in vitro* epithelium-macrophage model was used. The A549 adenocarcinoma human alveolar epithelial cells (Sigma Aldrich, Poole, UK) was used to model type II alveolar epithelium, whereas macrophages were primary human responder AMs isolated from healthy children by sputum induction.

2.9.1.1. Epithelial cell line

A549 adenocarcinoma human alveolar epithelial cells were grown in T75 cell culture flasks under standard growth conditions (i.e. 20 mL of complete Dulbecco's Modified Eagle Medium (DMEM) (F-12, Gibco, Thermo Fisher Scientific, USA) kept at 37°C, 5% CO_2 in humidified air in an incubator). Cells were passaged a maximum of 20 times. The A549 cell line was chosen because of its robust nature, enabling the cells to withstand a high dose of DEP, and is also a standard cell line used widely in respiratory research.

2.9.1.2. Choice of media and antibiotics

DMEM/F-12 is a basal medium widely used to support mammalian cell growth. DMEM/F-12 does not contain proteins, lipids, or growth factors, and therefore requires supplementation with FBS. The Ham's F-12 component of DMEM/F-12 is known to support epithelial cell growth. Complete DMEM was supplemented with

10% FBS and penicillin-streptomycin (Sigma-Aldrich, Missouri, USA), which was added to prevent bacterial (gram positive and negative) contamination.

Using the chamber wells as before, $1X10^3$ A549 cells per well were allowed to adhere overnight at 37°C, 5% CO_2, in complete DMEM. The cells were washed and chambers wells were resuspended with 150 µL of incomplete DMEM (supplemented with 4% FBS) and 150 µL of incomplete RPMI the following day. Chamber wells were divided into two groups: i) 10 µg/mL of DEP; and ii) 10 µg/mL of DEP and the entire population of enriched responder AMs previously extracted. Cells were exposed to DEP for 2 h before washing and staining. Light microscopy was used to determine the amount of black carbon associated with 50 randomly selected A549 epithelial cells from each culture – with and without primary AMs.

2.9.2. Effects of prostaglandin on the epithelium-macrophage model

To assess the effect of prostaglandin overproduction in the CF airways, PGE_2 was added to the epithelium-macrophage model. As before, $1X10^3$ A549 cells adhered to chamber wells overnight, before wells were washed and resuspended with 150 µL of incomplete DMEM and 150 µL of incomplete RPMI. Chamber wells were divided into three groups: i) 10 µg/mL of DEP; ii) 10 µg/mL of DEP and the entire population of enriched responder AMs extracted; and iii) 10 µg/mL of DEP, the entire population of enriched responder AMs previously extracted, and 10^{-6} M of PGE_2. PGE_2 was added to group (iii) 15 min before the 2 h exposure of DEP to all wells. Chambers wells were washed and stained for image analysis as above – 50 randomly selected A549 epithelial cells were analysed for black carbon content from each culture.

2.9.3. Cytokine release from epithelial cells following diesel exhaust particles exposure

IL-8 release from epithelial cells and AM was measured using an Enzyme-Linked Immunosorbent Assay (ELISA) assay.

For the trial run, the Duoset IL-8 ELISA kit (R&D systems, Minnesota, USA) was used. In accordance with the manufacturer's instructions, clear flat-bottom 92-well plate was pre-coated with capture antibody (100 µL/well at 4 µg/mL) overnight at 4^{o}C.

Standard wells were prepared by serial dilutions of the recombinant standard ("Top") well (1000 pg/mL) using the reagent diluent, generating 1:2 – 1:1024 dilutions.

Samples were tested in triplicate, in addition to negative and internal controls. Wells were washed with wash buffer (WA126, R&D systems) three times, flicking off the contents to ensure complete removal of capture antibody to safeguard good performance. Wells were then blocked (block buffer DY995, R&D systems) for 2 h at room temperature before the contents were flicked off and tapped dry. Samples and standards were added to wells and left for 2 h before washing, followed by addition of detection antibody for 2 h and washing. Working dilution of Streptavidin-HRP was added to each well in the dark for 20 min at room temperature before washing. Substrate Solution (1:1 Color Reagent A (H_2O_2) and Color Reagent B (Tetramethylbenzidine), DY999, R&D systems) were added to the wells in the dark for 20 min before stop solution (DY 994, R&D systems) was added to terminate the reaction. Optical density of each well is determined immediately after, using a

microplate reader set to 450 nm, with wavelength correction set to 540 nm. (i.e. subtract readings at 540 nm from readings at 450 nm).

The standard concentrations were adjusted according to the trial run standard curve (figure 2.12) – the highest 3 concentrations were omitted, subsequent standard concentrations started 1:8 of the original concentration (i.e. 125 pg/mL) (figure 2.13).

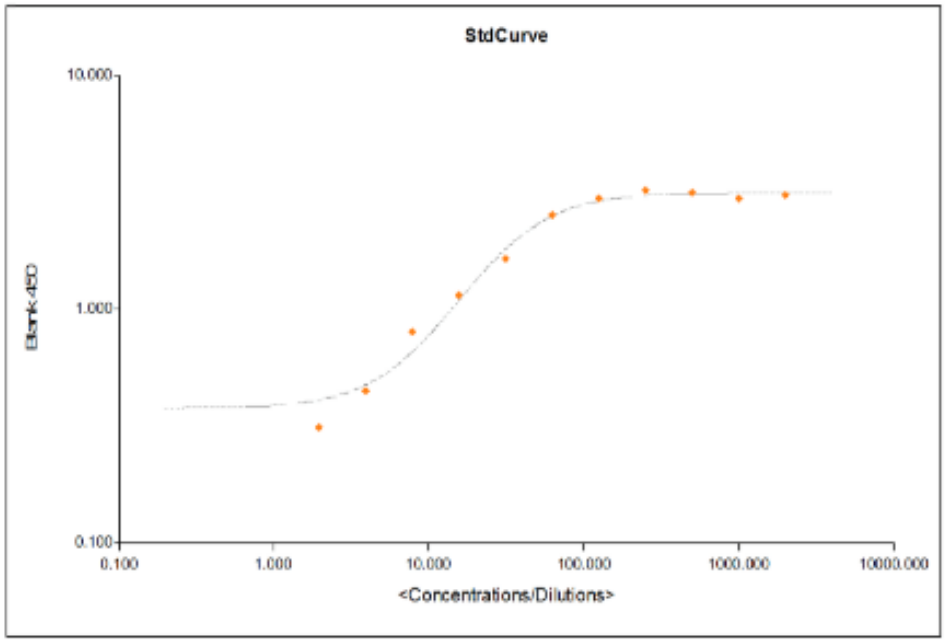

Figure 2.12. Standard curve using manufacturers' suggested concentrations. R^2 = 0.991.

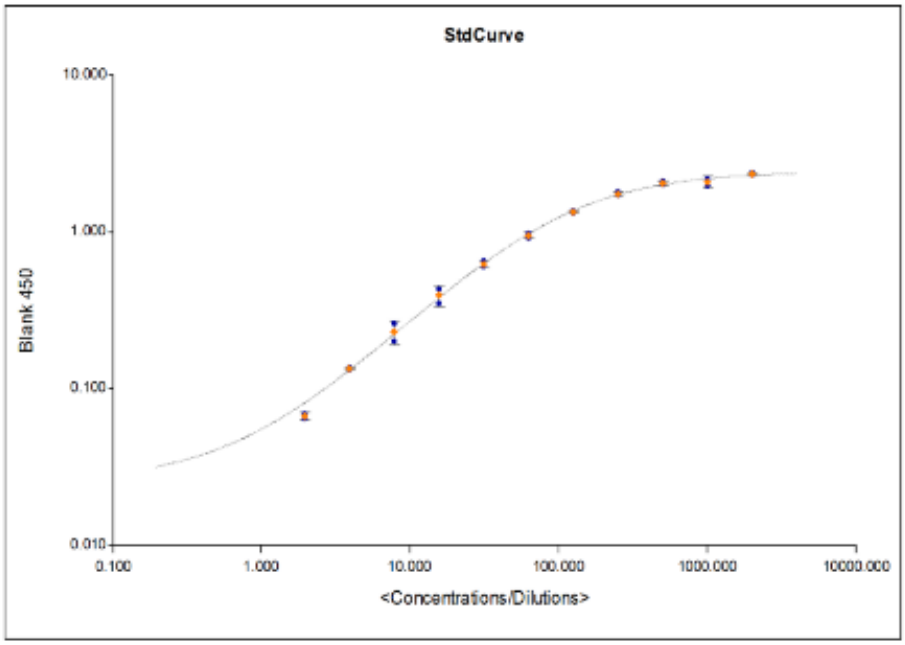

Figure 2.13. Standard curve using the modified standard concentrations. R^2 = 0.998.

However, the Duoset IL-8 kit was replaced by the Human IL-8 ELISA Kit (Invitrogen, California, USA) later in the project because it was less time consuming. Since experiments were performed in batches, it was deemed cost effective to use the pre-coated kit, where the microplate was pre-coated with target specific antibody. The kit was used according to the manufacturer's instructions. 7 serial dilution standards were used: 1000, 500, 250, 125, 62.5, 32.2, and 15.6 pg/mL, as well as blank wells containing only dilution buffer. Standards, blank controls and samples were added to the pre-coated wells and allowed for capture (immobilised) antibody binding, followed by the addition of detector antibody. A substrate solution was used to react with the enzyme-antibody-target complex, producing a signal which was proportional to the target concentration present. Absorbance reading was carried out at 450 nm. A standard curve was created using a curve-fitting software, subtracting the background absorbance. Values were adjusted by appropriate factor to correct for sample dilution (50 µL of each original sample was used). Duplicates

were used for each sample, mean values for each set of duplicates were determined.

2.9.3.1. Epithelial model

The A549 epithelial cell line was used to model the epithelium, $2x10^5$ A549 cells per well were allowed to adhere in a 24-well cell culture plate (Corning® Costar® , Corning, USA) overnight at 37°C, 5% CO2 in complete DMEM.

To compare the effects of DEP on the epithelial model, the wells were divided into two groups: i) unexposed controls; and ii) 10 µg/mL of DEP. The cells were washed the next morning, each well was resuspended with 1 mL of incomplete DMEM. DEP was added to group (ii) wells for 2 h. The plate was centrifuged at 1237 rpm (300 x g) for 5 min, the top 900 µL of supernatant was aspirated and stored at -80 ^oC until ELISA experiment, which was performed in batches.

Note: Methods of translocation of air pollution particles (research aim 1.10.1.2) can be found in chapter 6.

2.10. Statistical analysis

The initial aim of the project was to compare the personal exposure to air pollution and alveolar macrophage black carbon contents of 50 children with CF, with 50 age-matched healthy children. This would provide power of 90% at the 5% significance level to detect a difference between two means of 0.7 standard deviations, when comparing the CF and healthy control groups. However, expansion of the project (increased number of sputum inductions and experiments) resulted in fewer children recruited within the time frame, but statistically significant results were seen before reaching the original target.

Data were tested for normal distribution using the D'Agostino and Pearson omnibus normality test. Normally distributed parametric data are presented as mean ± standard error of the mean (SEM); non-parametric data are presented as median with inter-quartile range (IQR). Parametric data between 2 groups were compared using Student's unpaired or paired t-tests. Non-parametric data between 2 groups were compared by either Mann Whitney test (unpaired) or Wilcoxon matched-pairs signed rank test (paired). Comparisons between two observers or two analyses were done using Bland-Altman plots.

Multiple comparisons were performed using one-way ANOVA test, comparing means of difference ± standard error (SE).

Analyses were performed using Prism 8.00 for Windows (GraphPad Software, CA, USA). Results are considered significant at $p < 0.05$.

Chapter 3: *In vivo* and *in vitro* alveolar macrophage function in cystic fibrosis

3. *In vivo* and *in vitro* alveolar macrophage function in cystic fibrosis

3.1. Background

Air pollution has substantial harmful effects to various human body systems, with the lungs being most affected as the first point of contact after inhalation. Particulate matter ($PM_{2.5}$ and PM_{10}) and nitrogen oxide (NO_X) can cause oxidative stress and inflammation in the airways. Under normal circumstances, inhaled carbonaceous PM are phagocytosed by alveolar macrophages (AM) patrolling the lower airways. Phagocytosed particles are destroyed and removed from the airway, thereby reducing their accumulation and protecting other airway cells such as epithelial cells from their harmful effects. As discussed in section 1.7.1, the immune system in cystic fibrosis (CF) is altered, secondary to CFTR mutations. Not only does the presence of viscous airway mucus render mucociliary action challenging, the phagocytic ability of AMs is also impaired, leaving patients with CF susceptible to pathogens and inhaled pollutants. Impaired AM function will lead to less black carbon (BC) being phagocytosed, and accumulation of residual black carbon in the airways. Furthermore, organisms such as *Burkholderia cepacia* are able to invade, survive and replicate within macrophages, rendering their hosts susceptible.

3.2. Aims

In this chapter, the hypotheses tested are:

- Children with CF have reduced alveolar macrophage black carbon *in vivo* compared to healthy controls, despite being exposed to similar amount of air pollution.
- Alveolar macrophages from children with CF have reduced capacity to phagocytose particulate matter *in vitro*.

Personal exposure to air pollutants, particularly black carbon and nitrogen dioxide, in children with CF and healthy controls are compared. Using AMs obtained and enriched from induced sputum, the *in vivo* and *in vitro* AM function from both groups are assessed and compared.

3.3. Overall project: participants' demographics

3.3.1. Children with cystic fibrosis

36 children were approached at the Royal London Hospital, 2 did not wish to take part. 34 children with CF were recruited, 2 of whom withdrew after informed consent (1 participant found sputum induction challenging, 1 participant decided the study was too time consuming). Of the 32 enrolled children, 12 (38%) were male and 20 (62%) were female, age ranged from 2 to 16 years. Figure 3.1 gives an overview of patients' recruitment and participation in various parts of the study. Table 3.1 summarises the participants' CFTR mutations, most recent sputum or cough swab microbiology results at the time of recruitment, and lung function (FEV_1 predicted at the time of recruitment). The age and gender of participants are not shown in order to maintain pseudo-anonymity.

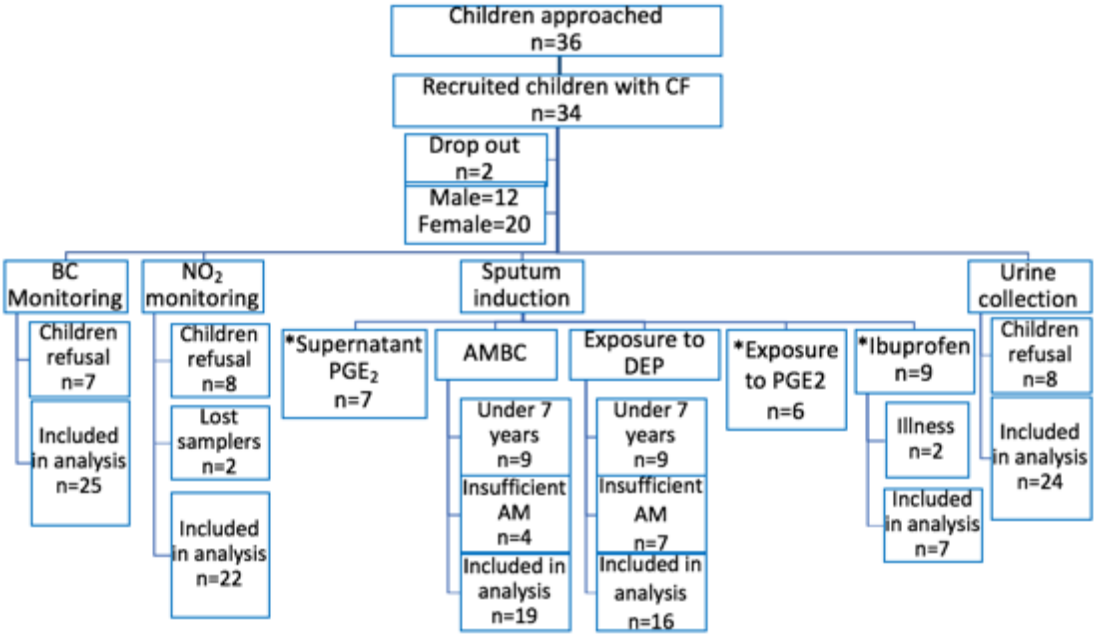

Figure 3.1. Flow diagram of CF patients' recruitment.

Patient	CFTR Mutations	Microbiology results at the time of recruitment	FEV1 predicted (%)
1	Delta F508: Delta exon 11 compound	Escherichia coli; Pseudomonas aeruginosa	N/A
2	Delta F508: N1303K	Staphylococcus aureus	76.8
3	Delta F508 homozygous	Staphylococcus aureus*	66.4
4	Delta F508 homozygous	Achromobacter xylosoxidans	73.8
5	Delta F508: R1158X	Throat flora	58
6	p.Gln685fs homozygous	No growth	N/A
7	Delta F508 homozygous	Aspergillus fumigatus	103.1
8	Delta F508 homozygous	Achromobacter xylosoxidans; Serratia	74.7
9	Delta F508 homozygous	No growth	N/A
10	p.CYS491Phe:CSER1118Phe	No growth	N/A
11	Delta F508: p.Arg352Gln	Throat flora	N/A
12	Delta F508 homozygous	Throat flora	N/A
13	Delta F508 homozygous	Yeast; Pseudomonas aeruginosa *	101.5
14	Delta F508: G551D	Haemophilus influenza; Burkholderia vietnamiensis	N/A
15	F549N homozygous	Throat flora	52.2
16	161Del C homozygous	Staphylococcus aureus*	73.9
17	Delta F508 homozygous	Pseudomonas aeruginosa*	N/A
18	Delta F508: c280s_2810del	No growth	81.6
19	Delta F508: R1158x	No growth	101.6
20	Delta F508 homozygous	Yeast; Throat flora	100.7
21	TRX4-10 del homozygous	Throat flora	90.5
22	Delta F508: parg158	Haemophilus influenza; Staphylococcus aureus	N/A
23	Delta F508 homozygous	Throat flora	97.1
24	Delta F508 homozygous	Stenotrophomonas maltophilia	102
25	Delta F508 homozygous	Throat flora	112.8
26	Delta F508 homozygous	Pseudomonas aeruginosa	70.1
27	Delta F508: 365insT	No growth	75.1
28	Delta F508 homozygous	Pseudomonas aeruginosa*	93.7
29	Delta F508: p.GLU60X	Throat flora	86.4
30	Delta F508 homozygous	Staphylococcus aureus; Aspergillus fumigatus	74.1
31	Delta F508 homozygous	Staphylococcus aureus; Scedosporium	70.2
32	Delta F508 homozygous	Stenotrophomonas maltophilia; Achromobacter xylosoxidans	92

Table 3.1. Summary of CF participants' CFTR mutation, most recent sputum or cough swab microbiology results at the time of recruitment, and lung function (FEV_1 predicted at the time of recruitment).

3.3.2. Healthy children

30 children were approached, and 4 did not wish to take part. 26 healthy children were therefore recruited as controls, 22 of whom were recruited at the Royal London Hospital, and 4 recruited in a school via media work; 1 was excluded after informed consent due to poor sputum induction technique. Of the 25 children, 11 (44%) were male, 14 (56%) were female, and ages ranged from 2 to 14 years. Figure 3.2 gives an overview of healthy children's recruitment and participation in various parts of the study.

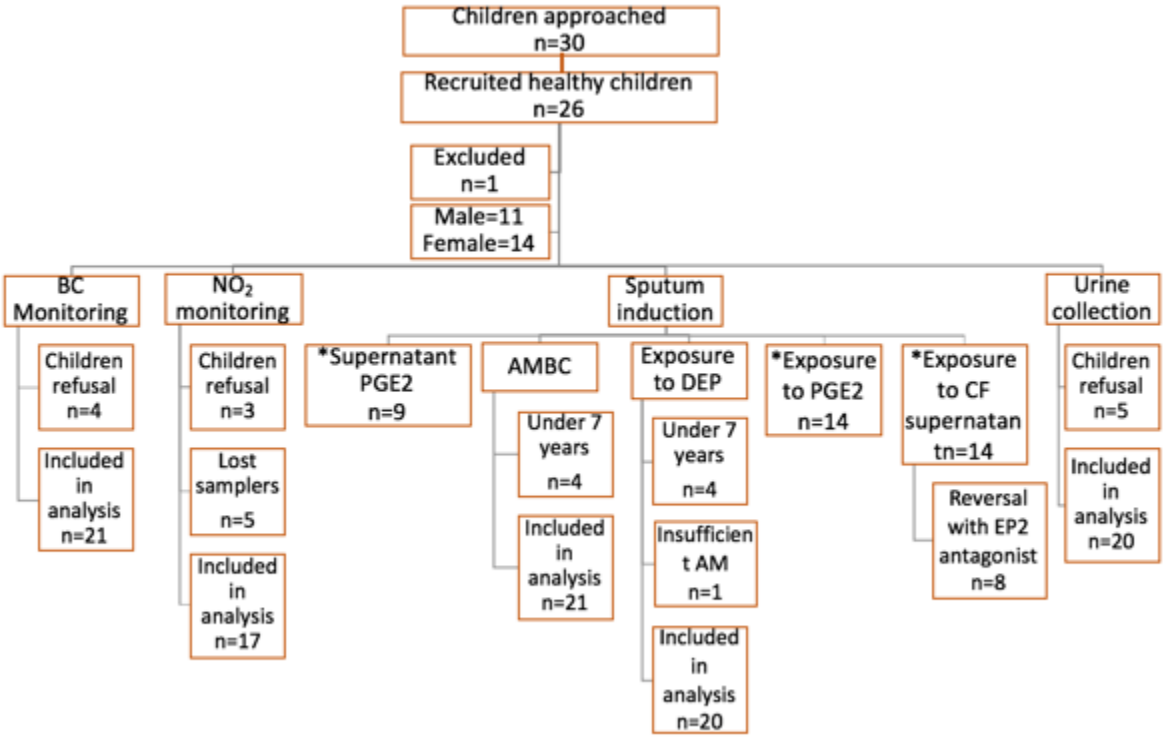

Figure 3.2. Flow diagram of healthy children's recruitment.

A total of 60 children were recruited following written informed assent (where old enough) and consent from the primary caregiver. Healthy controls were matched in age brackets to patients with CF, ranging from 2 to 16 years, median (IQR) ages of CF and control groups were 11.0 (4.0 – 15.0) years and 8.5 (7.0 – 11.0) years respectively (p=0.35, figure 3.3).

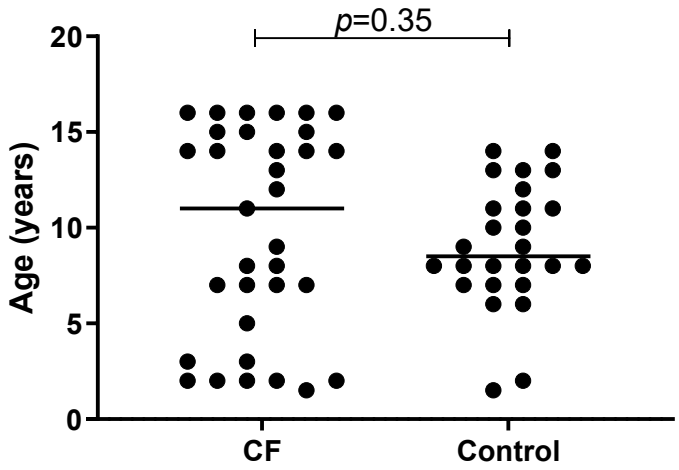

Figure 3.3 Age distribution of CF and control participants. Comparison by Mann-Whitney test. Bars represent median.

3.4. Pollution exposure monitoring

3.4.1. Modelled exposure at home address

Using the LAQT provided by King's College London, participants' pollution exposure 12 months prior to participation in the study was modelled based on their home address. There was no significant difference between the CF and control groups in terms of their long-term annual exposure to NO_2, PM_{10} and $PM_{2.5}$. Data are presented as median (IQR). Median NO_2 exposure for CF vs control groups were 34.84 (31.24 – 37.43) $\mu g/m^3$ vs 37.07 (32.14 – 40.96) $\mu g/m^3$ (p=0.09, figure 3.4). Notably, 4 participants from each of the two groups live in areas with annual NO_2 levels above the WHO guidelines.

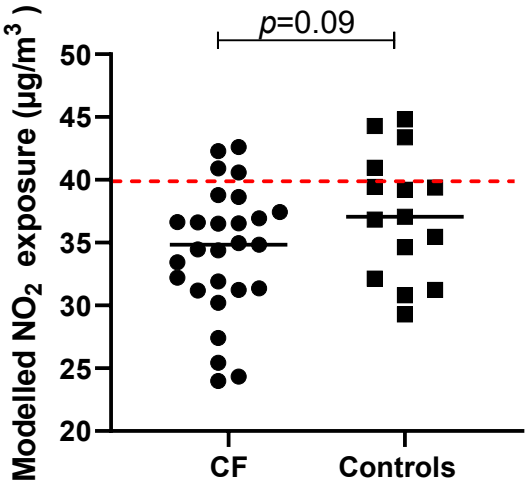

Figure 3.4. Modelled exposure to NO_2 of CF and control groups, 12 months before their participation in the study, based on their home addresses. Comparison by Mann-Whitney test. Bars represent median. The red line indicates WHO guidelines for annual NO_2 exposure.

Median PM_{10} exposure for CF vs control groups were 26.54 (24.53 – 27.26) µg/m^3 vs 25.41 (24.09 – 26.36) µg/m^3 (p=0.42, figure 3.5). All participants live in areas with annual PM_{10} levels above the WHO guidelines.

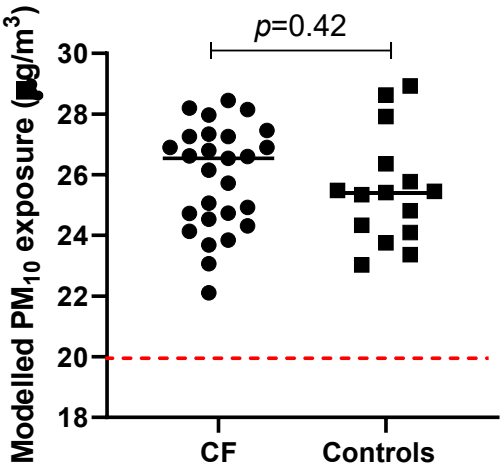

Figure 3.5. Modelled exposure to PM_{10} of CF and control groups, 12 months before their participation in the study, based on their home addresses. Comparison by Mann-Whitney test. Bars represent median. The red line indicates WHO guidelines for annual PM_{10} exposure.

Median $PM_{2.5}$ exposure were 16.81 (15.65 – 17.44) $\mu g/m^3$ vs 15.19 (14.65 – 16.86) $\mu g/m^3$ (p=0.22, figure 3.6). All participants live in areas with annual $PM_{2.5}$ levels above the WHO guidelines.

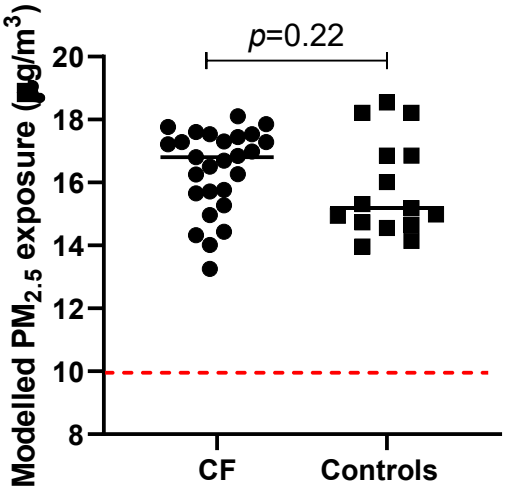

Figure 3.6. Modelled exposure to $PM_{2.5}$ of CF and control groups, 12 months before their participation in the study, based on their home addresses. Comparison by Mann-Whitney test. Bars represent median. The red line indicates WHO guidelines for annual $PM_{2.5}$ exposure.

3.4.2. Black carbon exposure

25 children with CF and 21 healthy controls took part in personal black carbon monitoring. Black carbon exposure was measured by portable aethalometers set to provide a reading every 60 s at a flow rate of 100 mL/min. Each participant carried an aethalometer for 2 typical school days, changing the filter every 24 h to ensure adequate device calibration.

Representative annotated graphs of two of the participants' aethalometer data are shown in figures 3.7 a and b. The baselines of the graphs are in the low-hundreds range, representing indoor levels of black carbon. Spikes are seen during participants' outdoor activities (e.g. commute and play-time).

To further examine the causes of high spikes, an aethalometer was carried along some of London's busiest roads (e.g. Oxford Street, Marylebone Road, The Strand), while the journey was filmed using a GoPro action camera. BC peaks were associated with idling vehicles or heavy vehicles (e.g. lorries, vans) passing by. Figure 3.8 shows some snapshots of the footage matched with the aethalometer BC peaks.

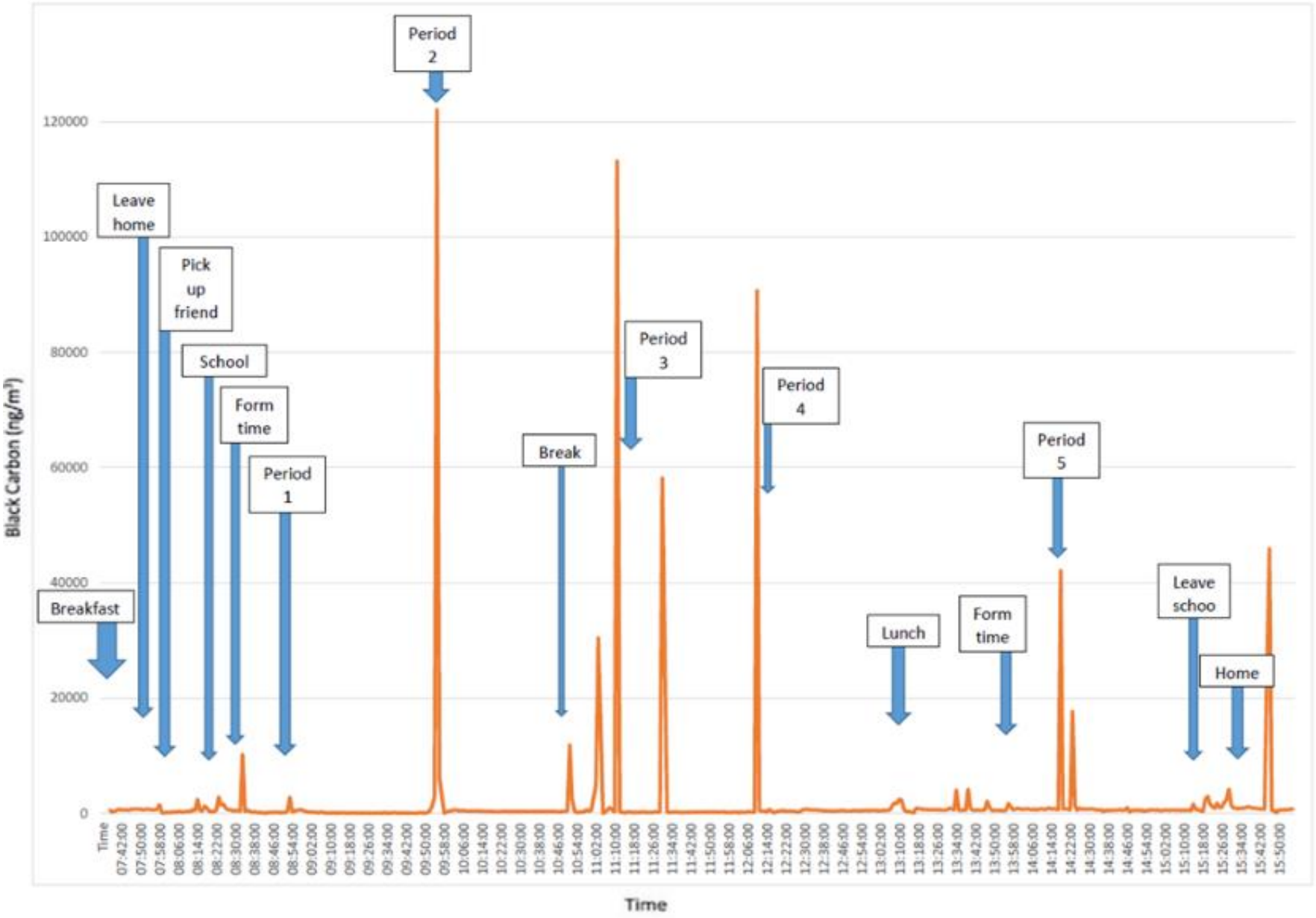

Figure 3.7a. Aethalometer reading of a participant's typical school day, showing spikes in black carbon levels during outdoor activities including walking to and from school, and walking between school buildings during lunch and in between periods.

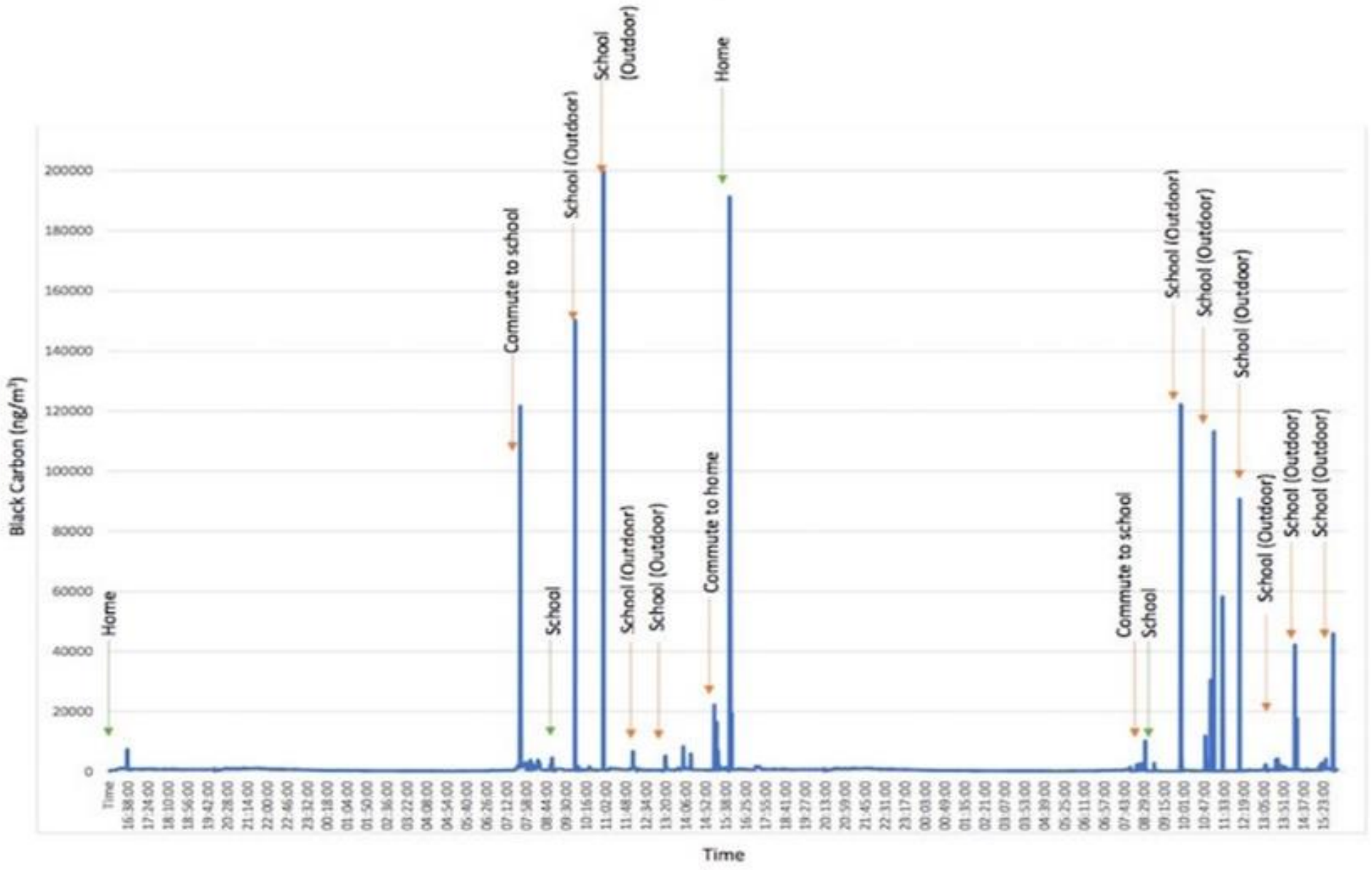

Figure 3.7b. Aethalometer reading of another participant's 2 typical school days, showing spikes of black carbon levels during outdoor activities including walking to and from school, and walking between school buildings during lunch and in between periods.

Figure 3.8. Aethalometer readings matched with GoPro camera footage to demonstrate real life environment (buses and lorries driving by) responsible for black carbon spikes.

The mean black carbon per minute across the 2 days for each child was determined by dividing the cumulative black carbon concentrations over the sampling period (2 days) by the number of sampling minutes. The median of these were compared between the two groups. In line with the modelled pollution data, there was no significant difference between the two groups. The median (IQR) of the mean black carbon per minute for CF and control groups were 1394 (828 – 3547) ng/m^3 (n=25) and 1587 (725 – 4392) ng/m^3 (n=21) respectively (p=0.83, figure 3.9).

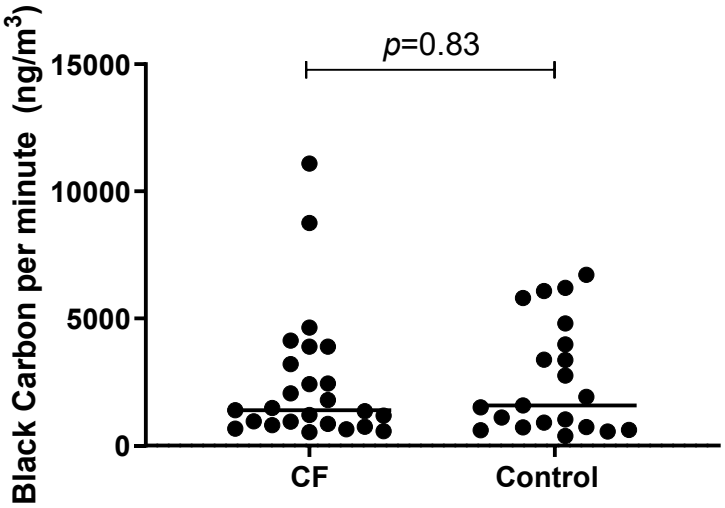

Figure 3.9. Personal black carbon exposure: each data point represents the mean per minute across 2 typical school days (cumulative black carbon concentrations of the 2 days divided by the number of sampling minutes). Comparison by Mann-Whitney test. Bars represent median.

The daily personal BC exposure data were shared with the participants in graphic form, in order to examine the pollution levels during their daily routine.

23 participants, combining CF and control groups, completed their activity diaries in detail. Their daily exposures to BC were categorised into 3 periods: i) home (indoor), ii) school (indoor) and iii) outdoor. The pollution peaks were identified; their cumulative BC exposure and time spent in each microenvironment was determined.

On average, children spent 23.5%, 66.4% and 10.1%, of their day indoors in school, home, and outdoors, respectively. The mean ± SEM daily cumulative BC concentrations were $1.4 \times 10^6 \pm 4.3 \times 10^5$ ng/m^3 (indoor school), $1.5 \times 10^6 \pm 2.1 \times 10^5$ ng/m^3 (indoor home), and $7.0 \times 10^5 \pm 1.9 \times 10^5$ ng/m^3 (outdoor). On average, the three microenvironments (school, home and outdoor) contributed to 29.6%, 52.0% and 18.4% of total daily BC exposure respectively (table 3.2). These results showed that children spent most of their time (66.4%) indoors at home, however, the cumulative BC exposure from this microenvironment was not significantly higher than the other two microenvironments. In fact, the cumulative BC concentration from home is similar to that from school, despite children spending approximately 3 times longer at home than in school. This suggested that the home environment was much cleaner than school. Similarly, while children only spent about 10.1% of the time outdoors (approximately one-sixth of their time at home), their cumulative BC concentration from the outdoor environment was almost half of that from home, suggesting the outdoor environment was the most heavily polluted amongst the microenvironments.

	School indoor	**Home indoor**	**Outdoor**
Mean duration (% of a day)	23.5%	66.4%	10.1%
Mean ± SEM of daily cumulative BC concentration	$1.4 \times 10^6 \pm 4.3 \times 10^5$ ng/m^3	$1.5 \times 10^6 \pm 2.1 \times 10^5$ ng/m^3	$7.0 \times 10^5 \pm 1.9 \times 10^5$ ng/m^3
Mean contribution of cumulative daily BC exposure	29.6%	52.0%	18.4%

Table 3.2. Summary of children's BC exposure in each microenvironment – combining data from both CF and control groups, only including children who completed their activity diaries in details.

3.4.3. Nitrogen Dioxide Exposure

NO_2 levels were measured using diffusive samplers. Taking into account the air flow and average temperature during the monitoring period, the mean NO_2 levels over 2 weeks were determined.

3.4.3.1. Indoor Nitrogen Dioxide Exposure

Indoor levels of NO_2 were measured by placing a diffusive sampler in a room in the house (except the bedroom) where the participant spent most time. Once again, there was no significant difference between the two groups. The mean indoor NO_2 over 2 weeks was determined for each child. The median (IQR) of was compared between the two groups: 23.45 (14.00 – 31.75) $\mu g/m^3$ standard temperature and pressure (STP) for CF group (n=22), and 24.80 (21.30 – 47.50) $\mu g/m^3$ STP for control group (n=17), p=0.21 (figure 3.10). It is unclear what was responsible for the CF outlier, an electric cook stove was used at home. For the control outlier, a gas cook stove was used and the kitchen was open-planned, the family burned candles 3 to 4 times a week; no other unusual activities were identified.

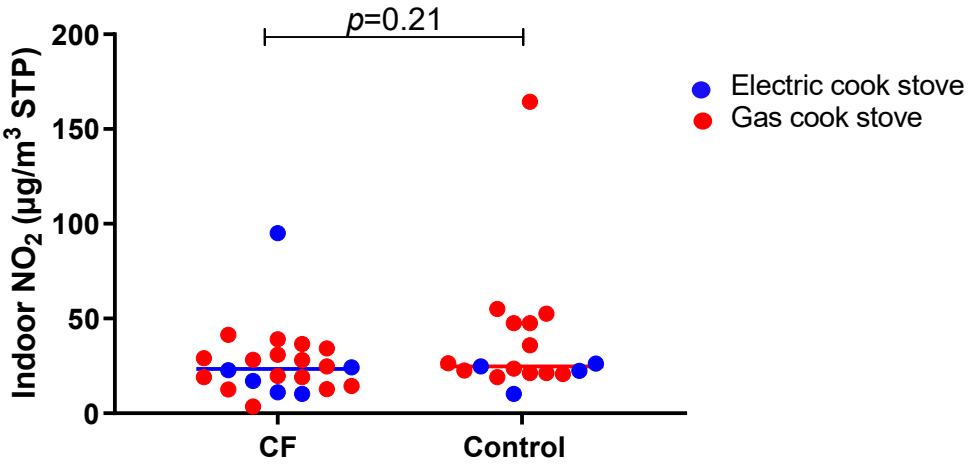

Figure 3.10. Indoor NO_2 levels: each data point represents the mean across 2 weeks for each participant. Comparison by Mann-Whitney test. Bars represent median.

There was no significant difference in indoor NO2 levels between participants (combined CF and controls) using gas (n=29) or electric (n=10) cook stoves, with median (IQR) of 24.80 (19.40 – 36.20) μg/m^3 STP vs 23.45 (15.40 – 29.48) μg/m^3 STP respectively, *p*=0.45 (figure 3.11).

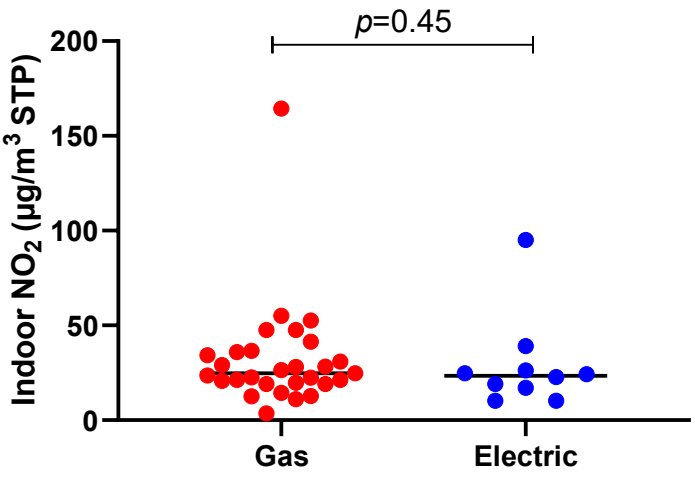

Figure 3.11. Indoor NO_2 levels for gas vs electric cook stoves, combining data from CF and control groups. Comparison by Mann-Whitney test. Bars represent median.

3.4.3.2. Personal Nitrogen Dioxide Exposure

Personal NO_2 exposure was measured by the participant wearing a diffusive sampler on their person during the 2 week monitoring period – a mixture of indoor and outdoor exposure. The calculations were based on a rough estimation of participants spending 4 h per day outdoor, and 20 h per day indoor. This estimation was kept constant for all participants. For the participants who did not damage or lose the NO_2 samples, the mean personal NO_2 exposure over 2 weeks was determined for each child. The median (IQR) of these were compared between the two groups: 19.60 (14.90 – 29.40) μg/m^3 STP for CF group (n=21), and 25.20 (17.33 – 36.00) μg/m^3 STP for control group (n=18), *p*=0.20 (figure 3.12). The CF outlier corresponds to the outlier for indoor NO_2 exposure in figure 3.10 in section 3.4.3.1.

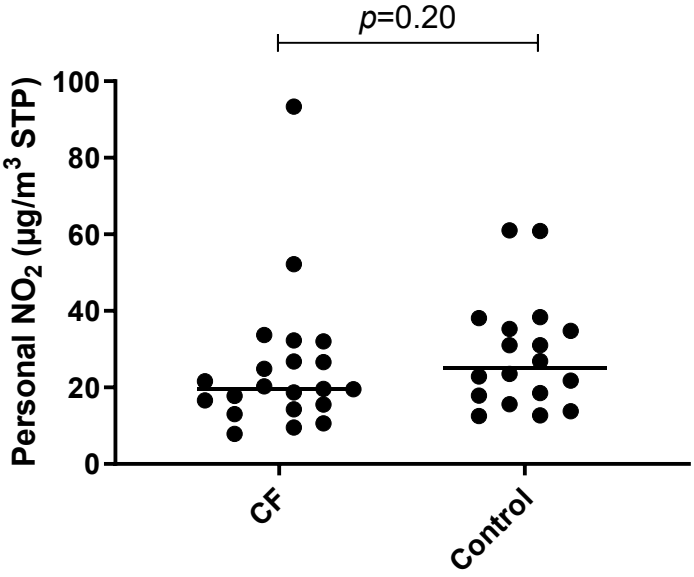

Figure 3.12. Personal NO_2 levels: each data point represents the mean across 2 weeks for each participant. Comparison by Mann-Whitney test. Bars represent median.

Using combined data from both CF and control groups (n=39), when the aethalometer readings (mean black carbon exposure per minute, over 2 typical school days) were compared to the personal NO_2 levels (mean NO_2 exposure over 2 weeks), no significant correlation was seen (r=0.28, *p*=0.08) (figure 3.13).

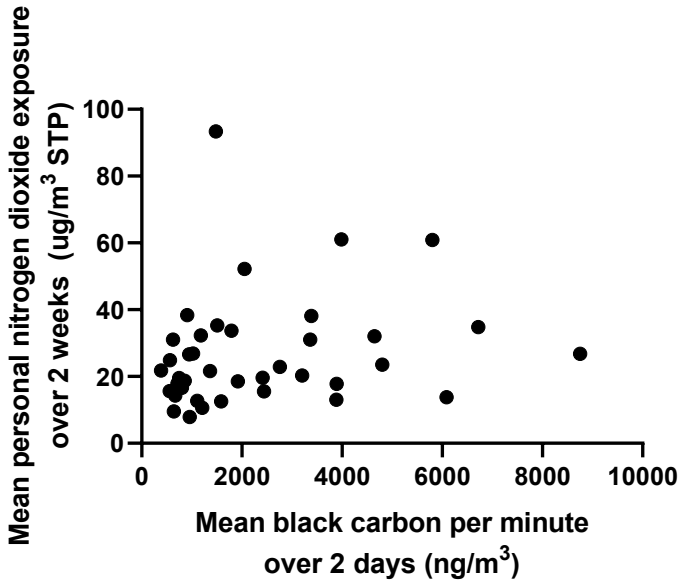

Figure 3.13. Spearman correlation between mean black carbon per minute over 2 days and mean personal nitrogen dioxide over 2 weeks, r=0.28, *p*=0.08.

3.5. Assessment of internal dose of air pollution: alveolar macrophage black carbon

Using image analysis of cytospin slides from both CF and control groups, mean alveolar macrophage black carbon (AMBC) of 50 randomly selected alveolar macrophages (AMs) from each participant was determined and the median of these were compared. AMBC of the CF group was significantly reduced compared to the control group, with median (IQR) of CF (n=19) vs controls (n=21) being 0.12 (0.05 – 0.24) μm^2 vs 0.30 (0.20 – 0.52) μm^2, $p<0.001$ (figures 3.14).

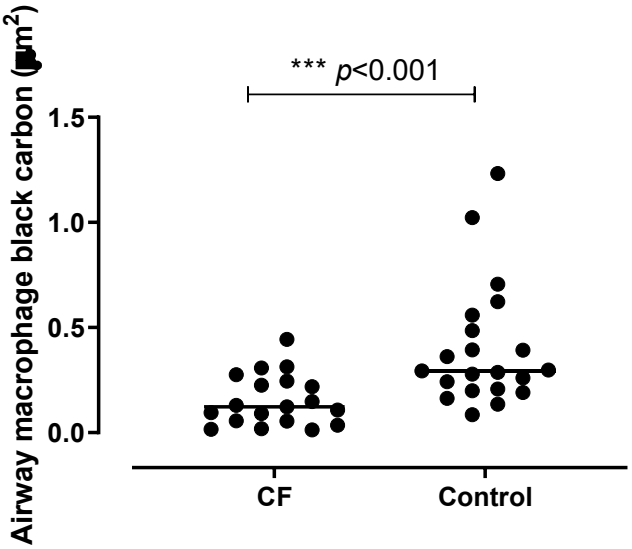

Figure 3.14. Alveolar macrophage black carbon (AMBC): each data point represents the mean of 50 randomly selected AMs from each participant. Comparison by Mann-Whitney test. Bars represent median.

Figure 3.15 shows the 50 AMs analysed from a randomly selected participant from the CF (figure 3.15a) and control (figure 3.15b) groups. Notably, there were more "clean" cells (i.e. cells with no black carbon within its cytoplasm) in the CF group compared to control group. There were also differences in cell morphology between the groups: CF AMs were generally smaller (as described in the literature), with more vacuoles within the cytoplasm, and cell membranes were more ragged and irregular – potentially due to imminent cell death. There were more prominent pseudopodia observed in CF AMs, possibly reflecting more active phagocytosis. These morphological differences suggest that CF AMs may be under more stress *in vivo*.

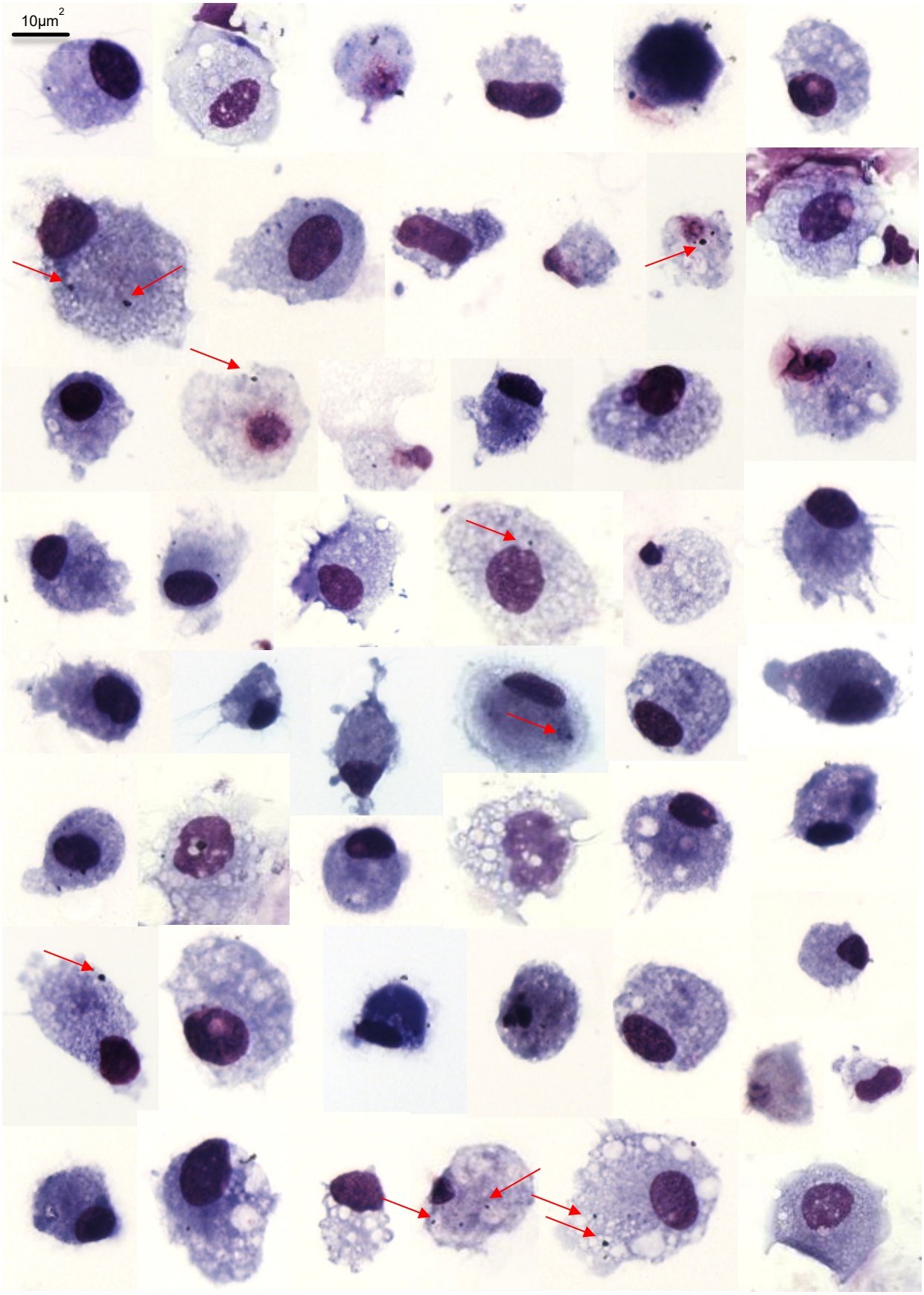

Figure 3.15a. 50 AMs with phagocytosed black carbon (arrows) from a CF participant - most cells contained no black carbon within the cytoplasm.

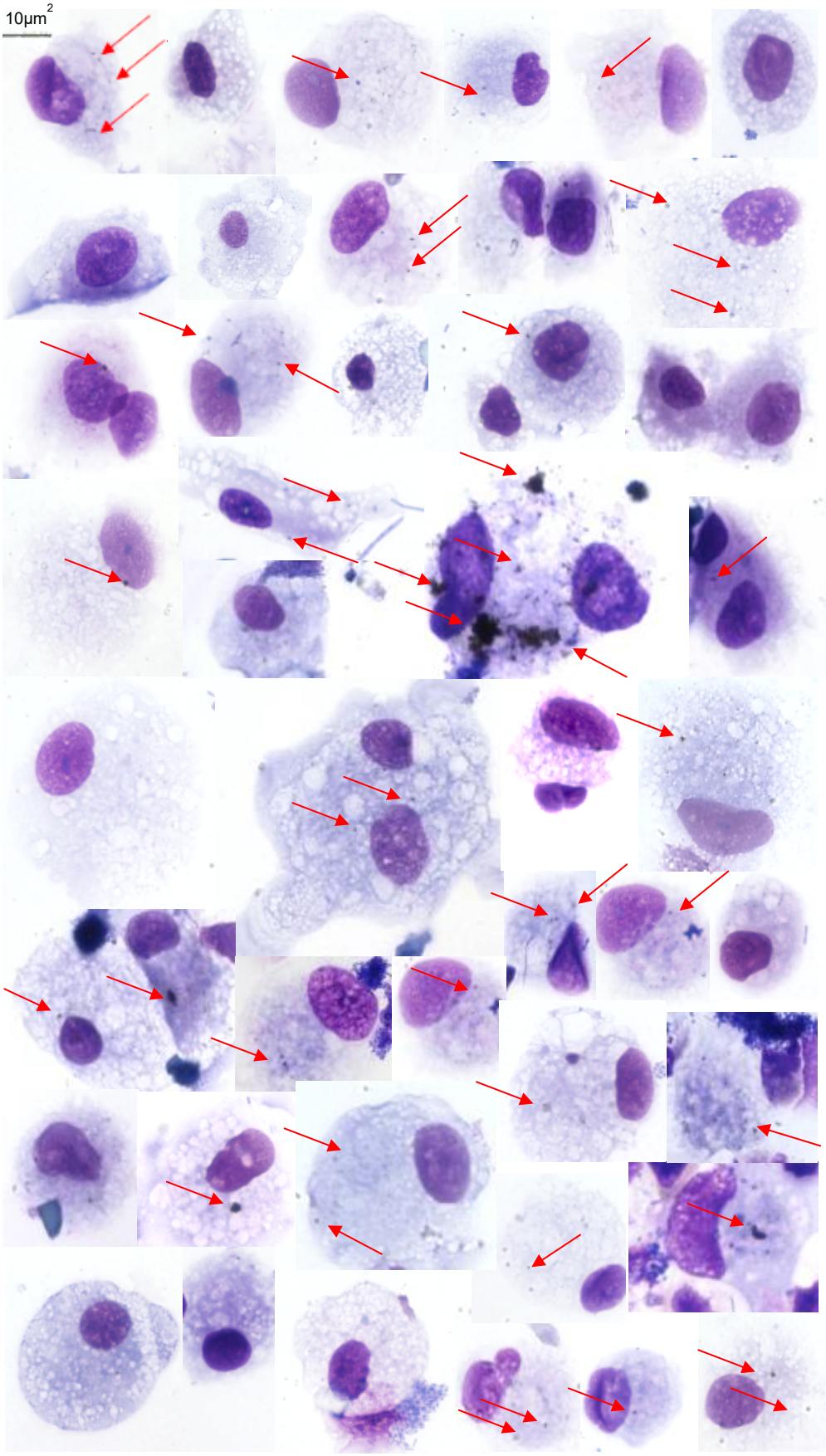

Figure 3.15b. 50 AMs with phagocytosed black carbon (arrows) from a healthy participant. More cells containing black carbon spots were seen.

3.5.1. Second observer for alveolar macrophage black carbon

Due to the temporal nature of participants' recruitment, where children from each group were recruited in batches, it was difficult to blind image analysis of AMBC. In order to limit unconscious bias, a second observer who was blinded to the participants' health status (CF vs control) performed the AMBC analysis on 11 randomly selected samples independently, followed by Bland-Altman analysis. Bias ± SD was 0.09 ± 0.19 with a 95% limits of agreement from -0.29 to 0.47 (figure 3.16).

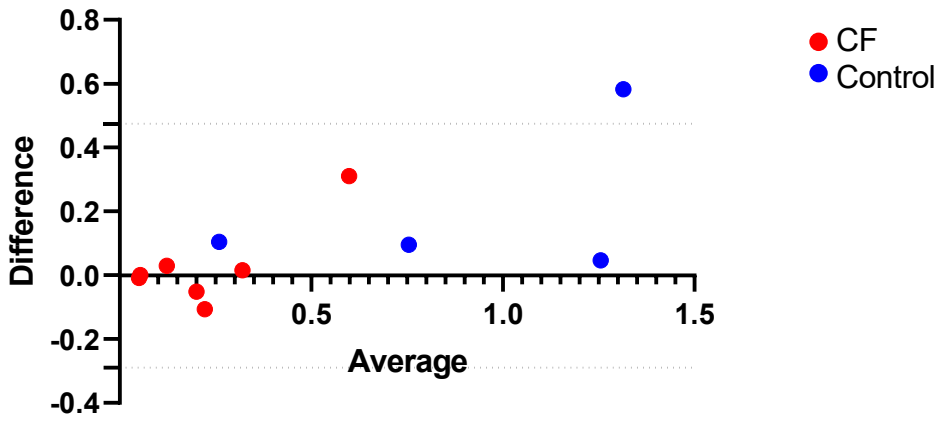

Figure 3.16. Bland-Altman: difference vs average. Bias and agreement of AMBC of 11 participants (CF and controls) by two independent observers – where the second observer was blinded to the participants' health status.

Generally, smaller differences are seen in the analyses of the CF group, where AMBC loading is reduced.

3.5.2. Relationship between alveolar macrophage black carbon and personal black carbon exposure

There was no significant correlation between AMBC and personal black carbon exposure (from aethalometer monitoring) for both CF (r=-0.51, *p*=0.06, figure 3.17a) and control (r=-0.18, *p*=0.49, figure 3.17b) groups.

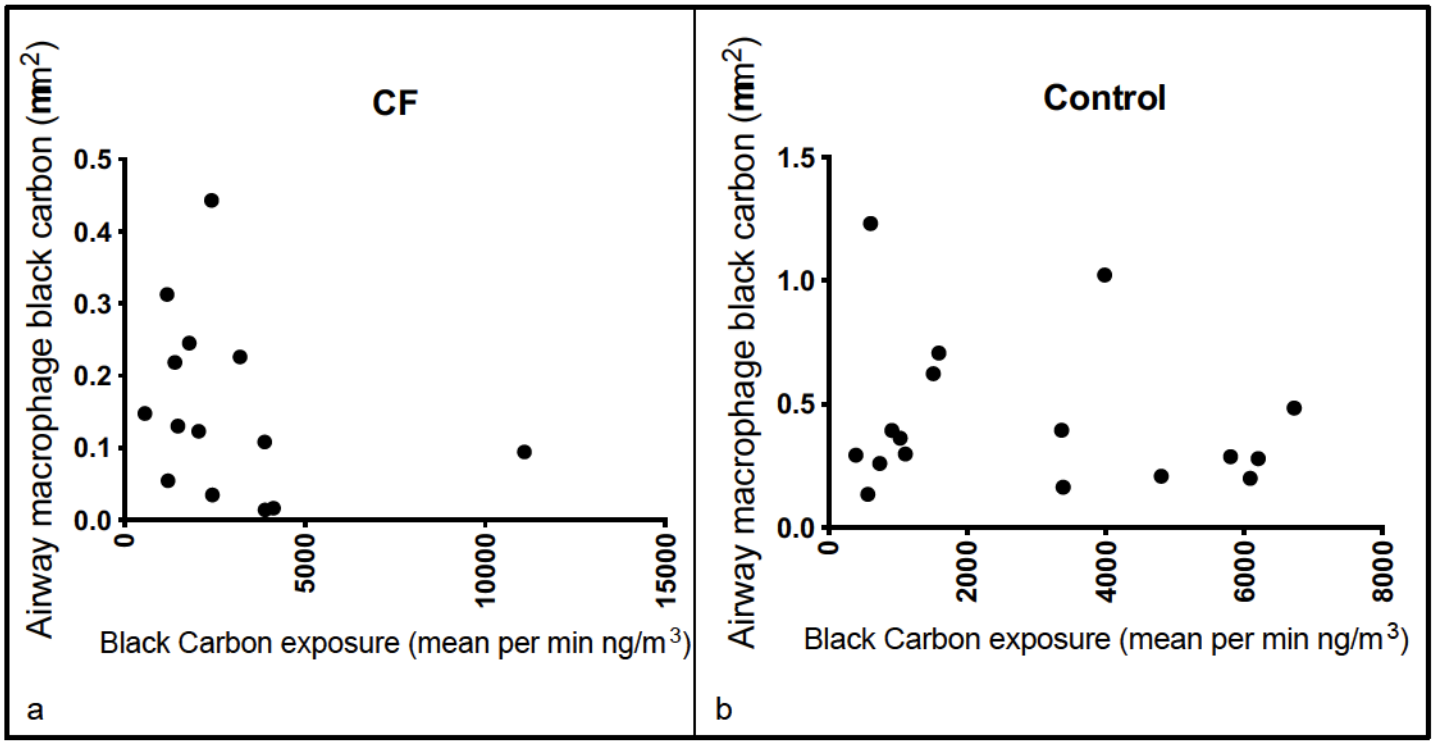

Figure 3.17. Spearman correlation between alveolar macrophage black carbon and mean black carbon per minute over 2 days for CF (a) and control (b) groups.

3.6. Alveolar macrophages *in vitro* uptake of diesel exhaust particles

Enriched AMs were exposed to 10 µg/mL of diesel exhaust particles (DEP) for 2 hr before AMBC analysis. It was difficult to achieve overnight cell adhesion of 50 cells per sample due to the variable and limited numbers of AMs extracted from each sputum induction session, coupled with inevitable cell loss during the enrichment process. Therefore, for each participant, equal numbers of AMs from each culture (unexposed vs exposed to DEP) were used for AMBC analysis. Table 3.3 depicts the number of AMs extracted from each participant by sputum induction and cell enrichment.

Participant (CF)	Number of cells per culture (unexposed vs DEP exposed)	Participant (Control)	Number of cells per culture (unexposed vs DEP exposed)
1	50	1	50
2	50	2	10
3	50	3	50
4	50	4	16
5	50	5	24
6	50	6	17
7	15	7	50
8	20	8	50
9	32	9	30
10	22	10	50
11	10	11	50
12	50	12	50
13	48	13	19
14	25	14	50
15	17	15	23
16	14	16	50
		17	27
		18	50
		19	24
		20	50

Table 3.3. Summary of the number of alveolar macrophages available from each participant after cell enrichment and overnight adhesion, for the DEP *in vitro* assay.

Comparing the number of AMs extracted from each participant, there was no significant difference between the CF and control groups. Median (IQR) number of AMs for CF and control were 40 (18 – 50) and 50 (23 – 50) respectively (p=0.49, figure 3.18).

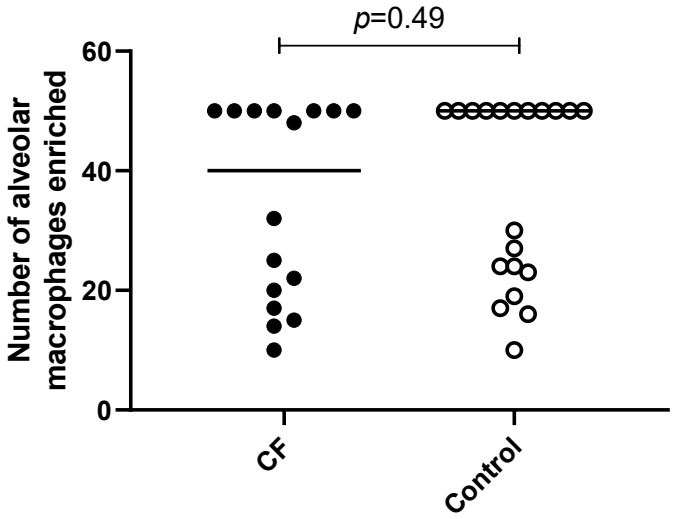

Figure 3.18. Number of alveolar macrophages available following cell enrichment and overnight adhesion for both CF and control groups. Comparison by Mann-Whitney tests. Bars represent median.

For the first time, image analysis of enriched primary AMs was performed in this project. The median of differences of AMBC for the CF group was 9.20 μm^2, p<0.0001 (n=16, figure 3.20a), increased by a median (IQR) fold change of 72 (48–238) (p<0.0001, figure 3.20c); the median of differences of AMBC for the control group was 11.53 μm^2, p<0.0001 (n=20, figure 3.20b), increased by a median (IQR) fold change of 40 (20 – 81) (p<0.0001, figure 3.20d).

Multiple comparisons showed a mean difference ± SE between CF unexposed cells and CF exposed cells of 13.81 ± 4.00 μm^2, p=0.003; a mean difference ± SE between CF exposed cell and control exposed cells of 0.11 ± 3.80 μm^2, p=1 (figure 3.20e).

These results demonstrate that DEP exposed cells from both CF and control groups contained comparable amount of black carbon, suggesting AMs from both CF and control groups had similar phagocytic ability *in vitro* (figure 3.19).

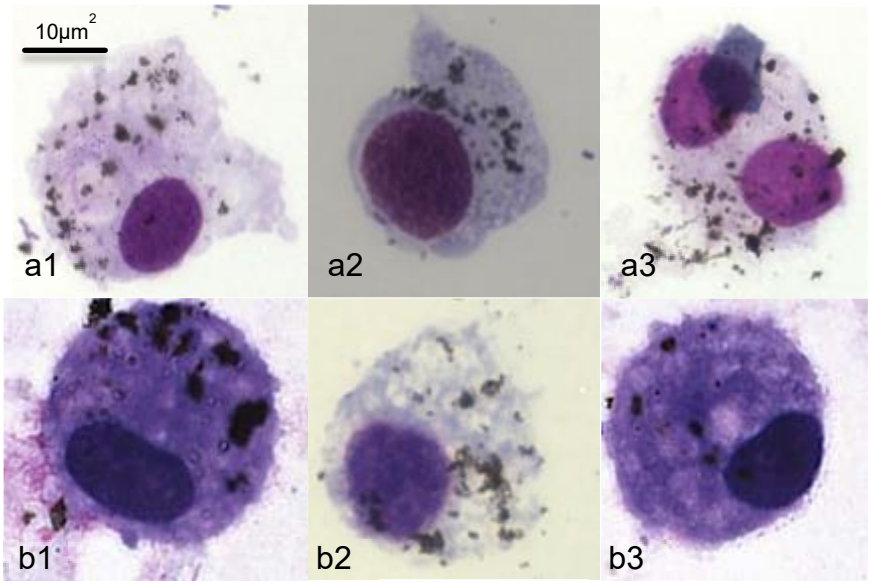

Figure 3.19. Alveolar macrophages from children with CF (a) and healthy controls (b), following exposure to diesel exhaust particles.

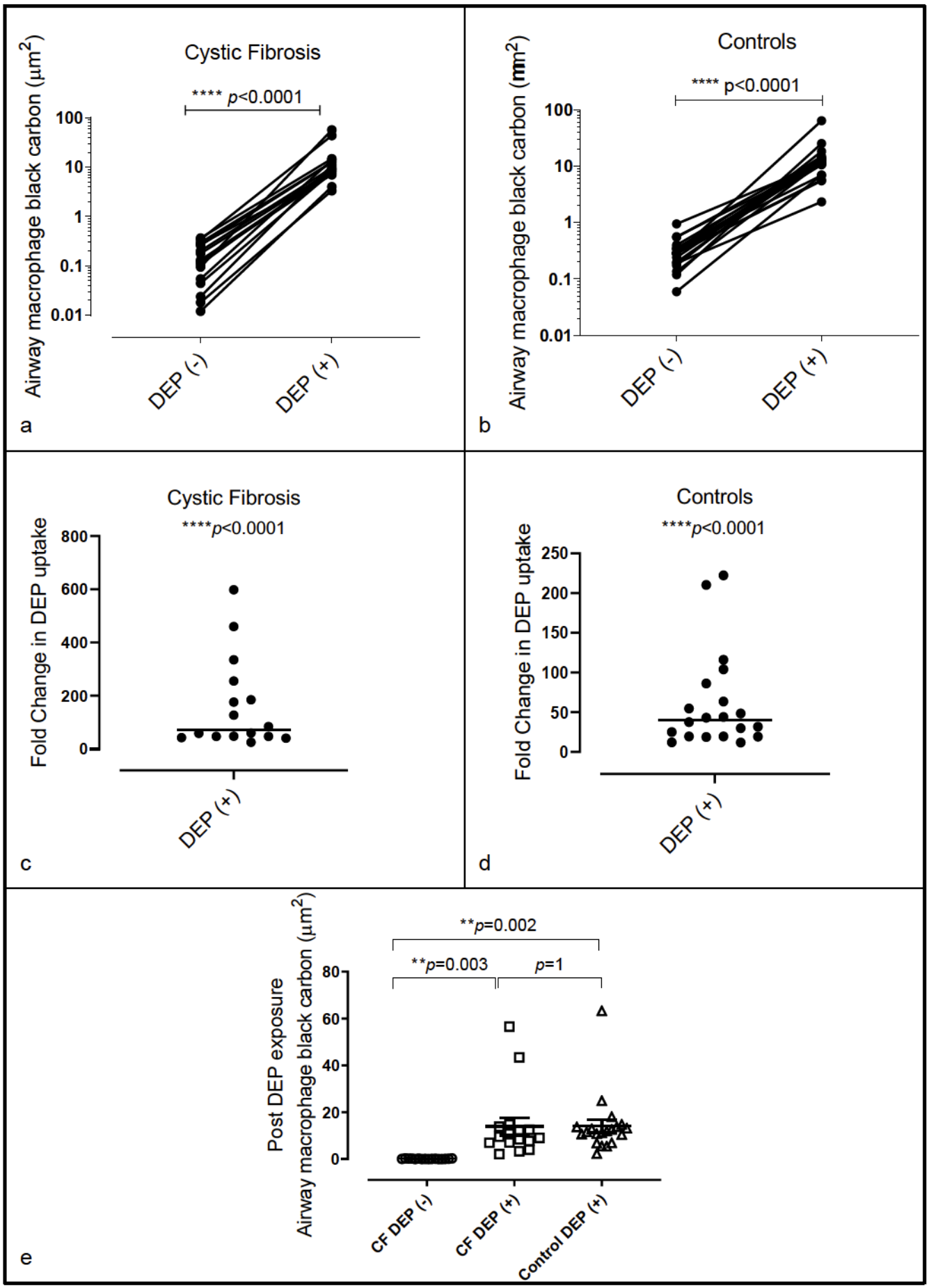
Cystic Fibrosis
**** p<0.0001
Airway macrophage black carbon (µm²)
100
10
1
0.1
0.01
DEP (-)
DEP (+)
a
Controls
**** p<0.0001
Airway macrophage black carbon (µm²)
100
10
1
0.1
0.01
DEP (-)
DEP (+)
b
Cystic Fibrosis
****p<0.0001
Fold Change in DEP uptake
800
600
400
200
0
DEP (+)
c
Controls
****p<0.0001
Fold Change in DEP uptake
250
200
150
100
50
0
DEP (+)
d
**p=0.002
**p=0.003
p=1
Post DEP exposure
Airway macrophage black carbon (µm²)
80
60
40
20
0
CF DEP (-)
CF DEP (+)
Control DEP (+)
e

Figure 3.20. Alveolar macrophage black carbon (AMBC) of alveolar macrophages extracted from CF (a) and control (b) participants, unexposed to diesel exhaust particles (DEP -) vs exposed (DEP+); presented in log scale and compared by Wilcoxon test. Data transformed into fold change in (c) and (d); compared by Mann-Whitney test, bars represent median. (e) Comparison of AMBC of unexposed (DEP -) alveolar macrophages from CF group, exposed (DEP +) alveolar macrophages from CF group, and exposed (DEP +) alveolar macrophages from control group; using one-way ANOVA Tukey's multiple comparisons test, bars represent SE.

3.7. Incidental findings - intracellular bacterial clusters

While many AMs examined, from both CF and control groups, contained scattered phagocytosed bacteria, it was noted that clusters of bacteria were occasionally seen in AMs from children with CF, a phenomenon not observed in the control group. 13 CF sputum samples were examined, using 50 randomly selected AMs from each sample. 5 of the 13 samples contained clusters of bacteria (rods or cocci, figure 3.21), with a mean ± SEM area occupied by bacteria of 2.41 ± 0.81 μm^2.

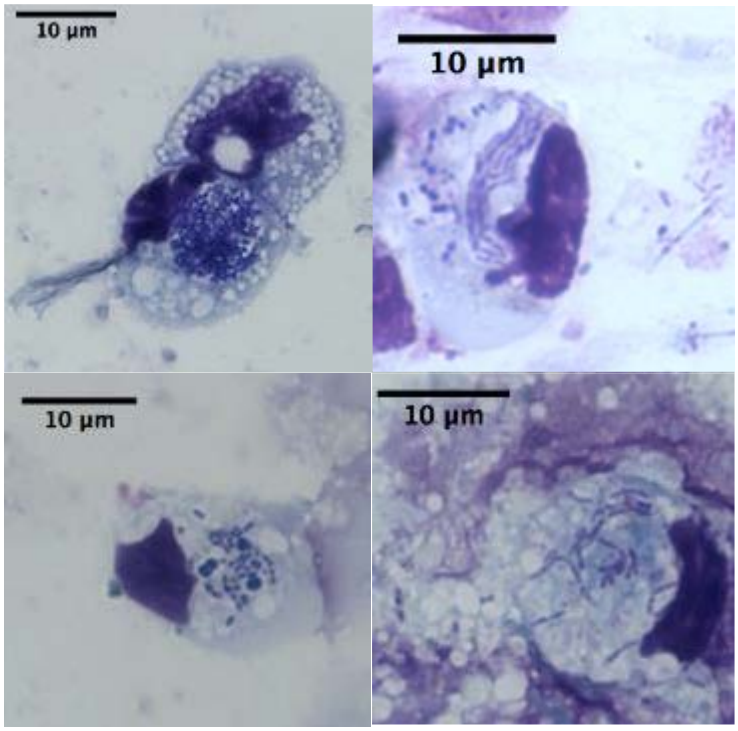

Figure 3.21. Alveolar macrophages from children with CF showing clusters of intracellular bacteria.

Of these 5 children, from their routine hospital sputum cultures at the time of research sputum induction, 3 samples yielded no growth, 1 child was colonised with *Pseudomonas aeruginosa*, and 1 was colonised with *Achromobacter xylosoxidans*.

In view of these incidental findings, further sputum samples from 2 of these children were obtained for repeat microbiology culture. Sputum induction was performed as

described in section 2.6.2. Half the sample was unprocessed and sent for prolonged routine hospital sputum culture. Briefly, an equal volume of 0.1% dithiothreitol was added to the sputum sample, followed by agitation and incubation at 37^{o}C for 30 min. 100 µL of the homogenised sputum was added to a sterile tube containing 900 µL of sterile distilled water. Serial 1:10 dilution was carried out down to 1/100000. Dilution concentrations of 1/10, 1/1000 and 1/100000 were plated on different media: blood agar, chocolate agar and Sabouraud Dextrose Agar. The other half of the sputum sample was processed (as per section 2.7.1.) in order to enrich for AMs, followed by microbiology culture as described above. Culture results are summarised in table 3.4. The significance of these results is discussed later in this chapter.

Patient	Enriched AM culture	Unprocessed sputum culture
1 Male Age 12	*Achromobacter xylosoxidans* **Capnocytophaga ochracea* **Neisseria flavescens* **Staphylococcus aureus* *Staphylococcus epidermidis* **Streptococcus mitis* *Streptococcus salivarius*	*Achromobacter xylosoxidans* *Actinomyces oris* *Staphylococcus epidermidis* *Streptococcus oralis*
2 Male Age 14	*Enterococcus faecalis* *Rothia mucilaginosa* **Staphylococcus epidermidis* *Streptococcus oralis*	*Candida albicans* *Enterococcus faecalis* *Paenibacillus glucanolyticus* *Rothia mucilaginosa* *Streptococcus oralis*

Table 3.4. Summary of induced sputum prolonged cultures results: enriched alveolar macrophages vs unprocessed sputum. * Organisms grown in enriched AM culture only.

3.8. Discussion

This chapter demonstrates clear evidence of reduced *in vivo* alveolar macrophage black carbon in the CF group compared to healthy controls. This does not appear to be due to differences in pollution exposure between the groups, as all participants were school children residing in London. Both modelled exposure and personal monitoring data showed that they were exposed to similar levels of pollution (NO_2, PM_{10}, $PM_{2.5}$, and black carbon – the core of PM). Therefore, the differences between the two groups are suggestive of functional phagocytic disparities, supporting the initial hypothesis of this project. That is, the lower amount of black carbon phagocytosed by AMs from children with CF implies impaired phagocytosis in this group. A similar phenomenon was seen by Brugha *et al.* [102] in children with moderate to severe asthma, who had 51% less AM carbon compared to healthy children. Macrophage dysfunction in CF is long established in the literature. For example, Brusica *et al*. [120] stated that CF macrophages can undergo phenotype change during disease processes, altering their ability to regulate inflammatory response, remove invading materials and repair tissue damage; while Alexis *et al.* [122] described altered CF phagocytes surface markers expressions, leading to impaired host defence and clearance of invading materials. It was therefore anticipated that there would be reduced levels of black carbon phagocytosed in AMs from children with CF.

Given that AMs from both groups exhibited similar *in vitro* phagocytic abilities when exposed to DEP, it is therefore reasonable to assume that impaired phagocytosis in CF is due to the unique environment of the CF airways. Viscous CF airway mucus might hinder AM movements, thereby limiting their access to and uptake of inhaled carbonaceous particles. Additionally, the pro-inflammatory environment in the CF

airway might have inhibitory effects on AM function – this will be explored in the subsequent chapter.

Traditional beliefs of airway inflammation in CF being caused by respiratory infections have changed over time – with airway inflammation now believed to be a primary event in CF, existing even without evidence of infections as early as fetal and infancy stages, as demonstrated by Hubeau *et al.* [179], when a larger population of AMs was seen in CF airways compared to healthy controls. Brennan *et al.* [180] also saw a larger number of AMs in bronchoalveolar lavages in children with CF compared to a non-CF group. These suggest that, as part of the disease process in CF, the absolute number of AMs are higher, in which case the reduced levels of AMBC could be explained by the BC load being shared amongst more AMs.

In this work, intracellular clusters of bacteria were incidentally observed in AMs isolated from children with CF, a phenomenon not seen in the healthy group. Given that these children were clinically well at the time of sampling, with no evidence of lung function decline, new onset respiratory symptoms or active chest infections, it was reasonable to assume that these organisms were residing within the host, concealed from the immune system – once again suggesting altered function of AMs in CF, and this could be problematic in CF. For example, *Burkholderia cepacia* is reported to survive inside phagocytic cells [131], and *Burkholderia cepacia* infection is particularly deleterious in CF as it is highly resistant to antibiotics. Another pathogen of concern in CF is non-tuberculous mycobacteria (NTM), prevalence of which has increased over the last 4 decades, potentially because of increased environmental exposures and antibiotic usage [181]. NTM can cause progressive lung damage and

require prolonged courses of antibiotic treatments, but diagnosis is often challenging due to its slow growth and the fact that NTM can exist naturally in the environment [182]. NTM isolation should therefore be ideally from sterile respiratory specimens, which are difficult to obtain in clinical settings. However, culturing the contents of enriched AMs will allow for detection of any concealed pathogens in an environment free from respiratory secretions and saliva, and can therefore be beneficial in the management of CF.

While patients with CF are often admitted to the hospital due to acute chest exacerbations, a portion of them will be asymptomatic and/or are admitted purely due to lung function decline. However, their sputum and cough swab sample cultures often yield no significant bacterial growth, in which case antibiotic treatments are empirical. With regards to prolonged sputum microbiology culture results in this research, whilst most organisms identified in enriched AM cultures but not unprocessed cultures were commensal flora found in the oral cavity (*Capnocytophaga ochracea*, *Streptococcus mitis*, *Streptococcus salivarius*), respiratory tract (*Neisseria flavescens*) and skin (*Staphylococcus epidermidis*, *Staphylococcus aureus*), some of these organisms could cause opportunistic infections in an immune compromised host. For example, *Streptococcus mitis* can cause infective endocarditis, and *Staphylococcus aureus* can cause problematic chest infections in CF, usually requiring antibiotic treatment. Therefore, using enriched AMs could offer a novel method of microbiology culturing, revealing any concealed pathogens and offering tailored antibiotic treatments.

3.8.1. Strengths

Aethalometers and diffusive samplers are gold standard methods of personal pollution monitoring, and are user friendly; whereas exposure modelling using the London Air Quality Toolkit is also well established. With participants from both CF and control groups being age-matched and all residing in London, their daily school activities and air pollution sources and levels were similar. This allowed for fair comparisons of AMBC findings – any differences in AMBC between the two groups were likely to be secondary to AM functional disparities. A second observer performing blinded image analyses on randomly selected samples from both groups helped maintain objectivity and reduce bias. Sputum induction is non-invasive and usually well tolerated, allowing for experimentations using primary AMs from both CF and control groups. The AM enrichment method allows for clear visualisation of AM content, and experimentations with primary AMs, without the influence of other airway cells and mucus.

3.8.2. Limitations

Personal exposure monitoring was only carried out for a limited period of time (2 days for BC monitoring, and 2 weeks for NO_2 monitoring). Air pollution levels change with season and weather (temperature, wind speed, humidity, etc). 8 aethalometers were available for use during the study period, as a result, participants were monitored on different school days, spanning across all seasons and weather variations.

Aethalometer use was limited by its battery life of up to 24 h. As a result, varying durations of data were obtained from each participant (ranging from 20 to 24 h per monitoring day). Data were therefore expressed as mean BC level per minute across

the monitoring period. Data interpretation relied heavily on participants' accurate recording of their activities in the diary, the quality of recordings were variable. NO_2 diffusive samplers were small and prone to loss and damage. Consequently, not all participants completed the NO_2 monitoring exercise.

It was challenging to quantify and compare the amount of AMs present in the CF vs control airways, as the AM yield from sputum induction was not directly proportional to their *in vivo* quantities. Yield was very dependent on the participant's induction technique and could vary even within the same individual on different occasions. As previously discussed, the variable absolute numbers of AMs in CF and controls could affect the interpretation of AMBC results.

While sputum induction is a non-invasive and well tolerated, techniques vary amongst children, resulting in differing amount of AMs extracted from each participant. This was particularly troublesome in the CF cohort, as CF airway secretions are viscous, making cell expectoration difficult; many patients have been labelled as "dry" and have struggled to expectorate samples over the years, whilst others tend to swallow sputum. As a result, the *in vitro* work was limited by the number of AMs available from each participant at each sputum induction session.

3.8.3. Summary

This chapter demonstrated that despite exposure to similar levels of air pollution, there was less phagocytosed black carbon *in vivo* in AMs isolated from children with CF, compared to their healthy counterparts. However, the AM phagocytic ability from both groups was similar *in vitro*. Putative explanations include the presence of

phagocytic-inhibitory substances within the CF airway, or an increased amount of AMs in CF, sharing out the load of inhaled BC. Nevertheless, the bacterial clusters seen in CF AMs, along with evidence in the literature, suggest AM function in CF is altered, thereby supporting the former explanation. The next chapter will discuss the cause of impaired AM function in CF, and in particular the potential role of prostaglandin, as seen in children with moderate to severe asthma.

Chapter 4:
Role of prostaglandin E2 in alveolar macrophage function in cystic fibrosis

4. Role of prostaglandin E2 in alveolar macrophage function in cystic fibrosis

4.1. Background

The previous chapter demonstrated that despite exposure to similar levels of air pollution, black carbon loading was reduced in alveolar macrophages isolated from children with cystic fibrosis, compared to their healthy counterparts. One obvious explanation is phagocytic impairment in CF. As highlighted in section 3.8, this could be due to altered alveolar macrophage morphology and size, and reduced receptor expression; it could also be due to the presence of a larger population of alveolar macrophages in CF, so the inhaled black carbon load is shared between more phagocytes. However, it is well recognised that inflammation characterises the airways of patients with CF. As discussed in section 1.7.2, there is increased recruitment of inflammatory cells, and increased production of cytokines and prostaglandins within the CF airways; CFTR dysfunction can also affect macrophage polarisation, contributing to inflammatory responses. Inflammation is particularly worse during exacerbations, but can also be present when patients are clinically well without any evidence of infections.

4.2. Aims

In this chapter, the hypotheses tested are:

- Children with CF have higher levels of prostanoids – specifically, increased levels of prostanoids and metabolites in the respiratory secretions and urine.
- Prostaglandins, specifically PGE_2, exert inhibitory effects on alveolar macrophage phagocytosis.
- There is increased expression of cyclooxygenase-2 in alveolar macrophages isolated from children with CF.
- Prostanoid production can be reduced by cyclooxygenase inhibitors (e.g. Ibuprofen).

Firstly, *in vivo* markers of inflammation including cyclooxygenase 2 (COX-2) and prostaglandins (PGs) metabolites were measured and compared in both CF and control groups. COX-2 expression in AMs was measured using flow cytometry, while urinary metabolites of prostanoids were used as a reflection of endogenous PGs synthesis due to its chemical instability, as discussed in section 2.8. Secondly, the inhibitory effect of prostaglandin, in particular PGE_2, on AM function was modelled *in vitro*; along with the demonstration of how PGE_2 blockade with an EP2 antagonist could restore AM phagocytosis. To further demonstrate the association, the effect of CF airway secretions on responder AM function (cells isolated from healthy controls) was also modelled.

4.3. Cyclooxygenase 2 expression in alveolar macrophages

Relative median fluorescence intensities (MFI) of COX-2 expression in enriched AMs were higher in the CF group (n=20) compared to the control groups (n=17). Median (IQR) MFI was 10217 (8092 – 16126) vs 8142 (6536 – 10209) for CF and control respectively, *p*=0.02 (figure 4.1).

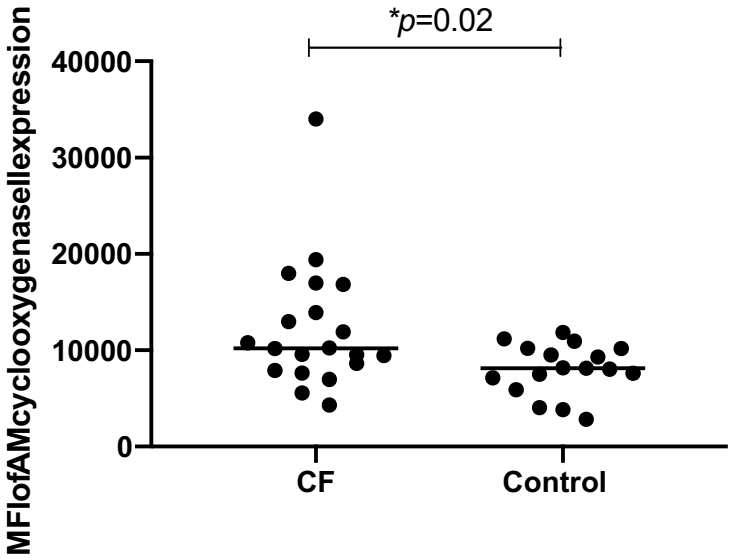

Figure 4.1. Median fluorescence intensities (MFI) for COX-2 expression in alveolar macrophages isolated from CF and control groups. Comparison by Mann-Whitney test. Bars represent median.

4.3.1. Blinded analysis of COX 2 expression in alveolar macrophages

8 samples (mixed CF and control groups) were randomly selected, different sample identification numbers were re-assigned by an independent researcher, thereby blinding the analyst from the participants' health status, followed by re-analysis by the original analyst (myself), using the same flow cytometry gating strategy. The bias and agreement of the median MFI for COX-2 expression from the two sets of

results were compared by Bland-Altman method. Bias ± SD was 1936 ± 8399 with a 95% limits of agreement from -14527 to 18399 (figure 4.2).

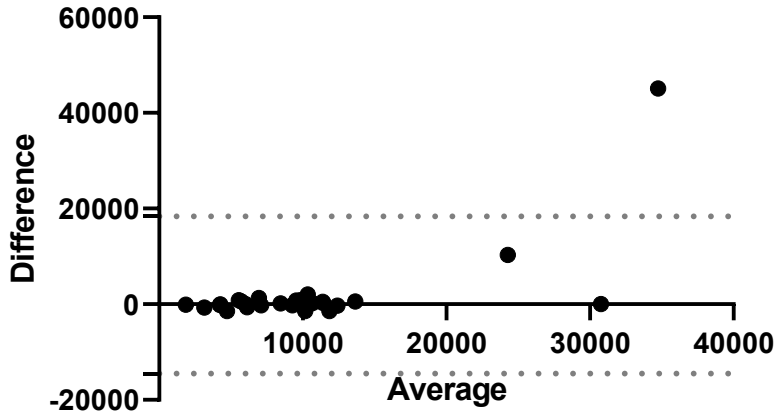

Figure 4.2. Bland-Altman: difference vs average. Bias and agreement of median MFI for COX-2 expression in AMs from 8 participants (CF and controls) by the same observer, blinded vs unblinded to the participants' health status.

4.4. Prostanoid profiles in cystic fibrosis compared to healthy controls

4.4.1. Indirect measurement of prostanoid production – urinary profiles

Urinary metabolites of prostaglandin E2 (13,14-dihydro-15-keto-E2; 13,14-dihydro-15-keto-tetranor-PGE2 and tetranor-PGE-M), prostaglandin D2 (13,14-dihydro-15-keto-tetranor-PGD2, tetranor-PGD-M, and 9a,11b-PGF2), and prostaglandin J2 (15-doexy-delta12,14-PGJ2) were higher in the CF group (n=24) than the control group (n=20), after correction for urinary creatinine levels (median (IQR) creatinine for CF and controls were 4987 (1847 – 8478) µmol/L vs 12291 (8227 – 16559) µmol/L respectively, $p<0.001$).

4.4.1.1. Prostaglandin E2

For urinary metabolites of PGE_2, median (IQR) urinary 13,14-dihydro-15-keto-E2 were 1490 (773 – 2675) pg/mg creat vs 928 (478 – 1376) pg/mg creat ($p=0.04$, figure 4.3).

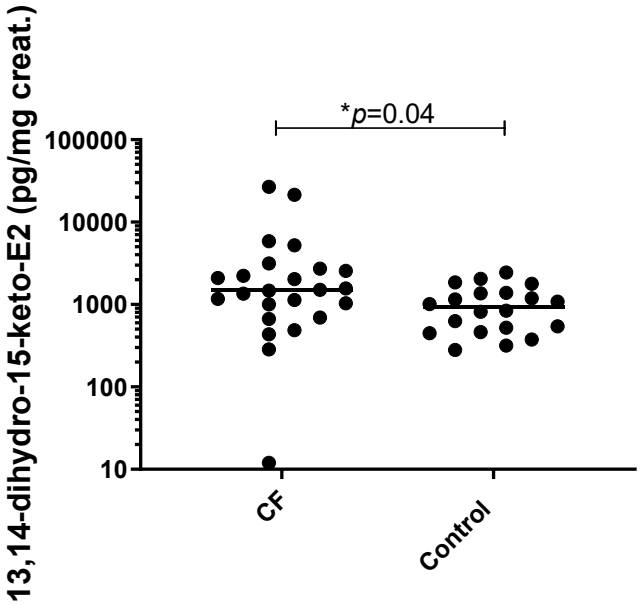

Figure 4.3. Urinary metabolite of prostaglandin E2: 13,14-dihydro-15-keto-E2 for CF and control groups, presented in log scale. Comparison by Mann-Whitney test. Bars represent median.

Median (IQR) urinary 13,14-dihydro-15-keto-tetranor-E2 for CF vs controls were 511 (249 – 788) pg/mg creat vs 214 (96 – 323) pg/mg creat (p=0.003, figure 4.4).

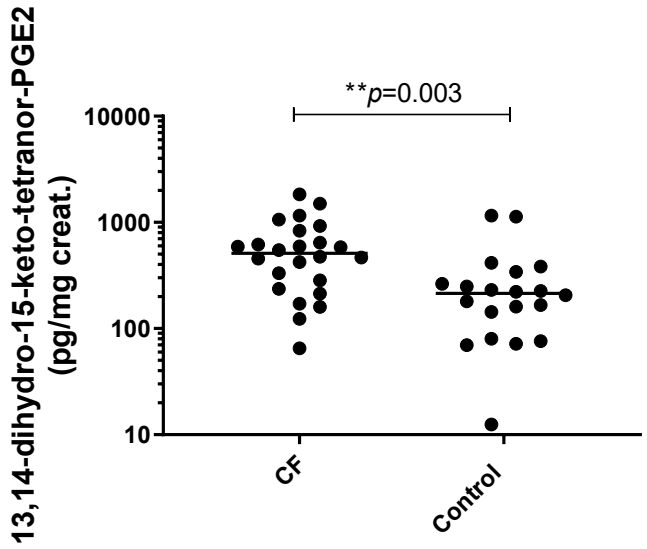

Figure 4.4. Urinary metabolite of prostaglandin E2: 13,14-dihydro-15-keto-tetranor-E2 for CF and control groups, presented in log scale. Comparison by Mann-Whitney test. Bars represent median.

Median (IQR) urinary tetranor PGEM, the main metabolite of PGE_2, were 35965 (22915 – 101316) pg/mg creat vs 15873 (10150 – 24733) pg/mg creat (p<0.001, figure 4.5).

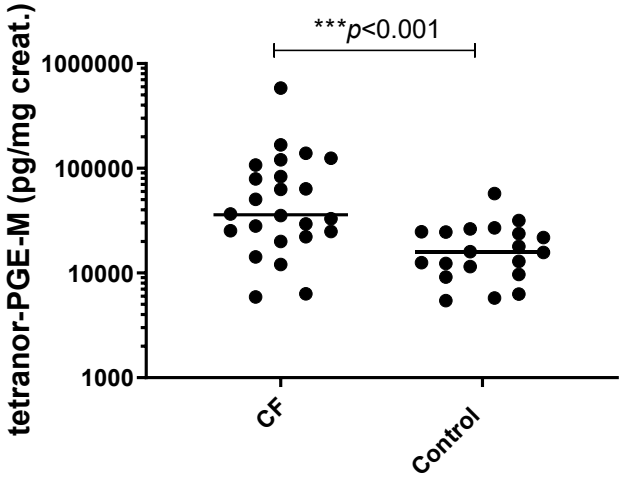

Figure 4.5. Urinary metabolite of prostaglandin E2: tetranor-PGE-M for CF and control groups, presented in log scale. Comparison by Mann-Whitney test. Bars represent median.

Since airway inflammation can progressively deteriorate with time in CF, the main metabolite of PGE_2 (urinary tetranor-PGE-M) was examined further in relation to the participants' age. All participants were clinically stable at the time of sampling. There was no relationship between urinary tetranor-PGEM and age of the children from the CF group (r=0.16, *p*=0.50) (figure 4.6).

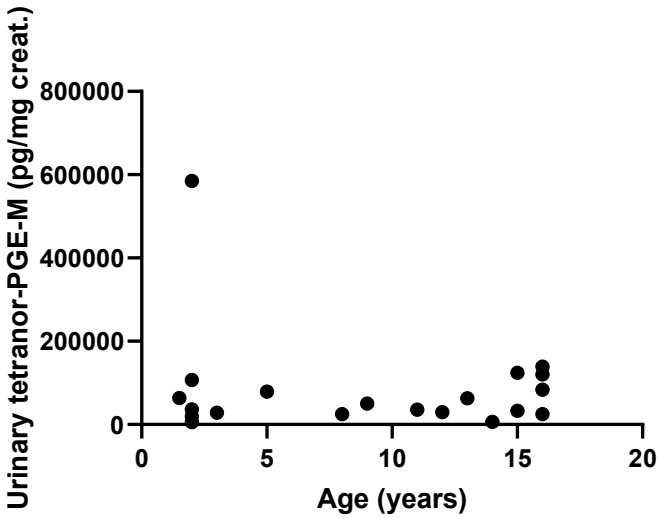

Figure 4.6. Spearman correlation: relationship between urinary tetranor PGEM and age of children with CF, r=0.16, *p*=0.50.

Lung function also deteriorates over time in CF, therefore, urinary tetranor-PGE-M levels of children with CF were compared to their spirometry results at the time of sampling, while clinically stable. There was no apparent relationship between urinary tetranor-PGE-M and FEV_1 predicted (r=0.26, *p*=0.42), (figure 4.7); nor was there a relationship between urinary tetranor-PGE-M and FVC predicted (r=0.35, *p*=0.21), (figure 4.8).

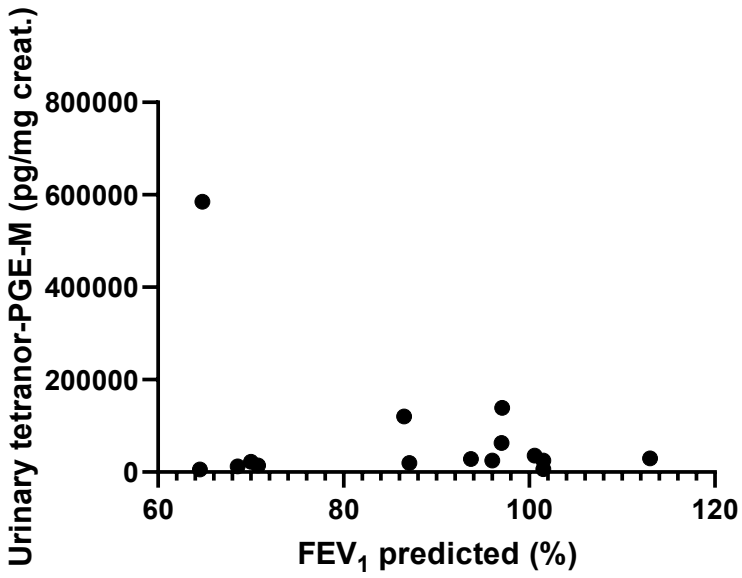

Figure 4.7. Spearman correlation: relationship between urinary tetranor-PGE-M and FEV_1 predicted (%) in children with CF, r=0.26, *p*=0.42.

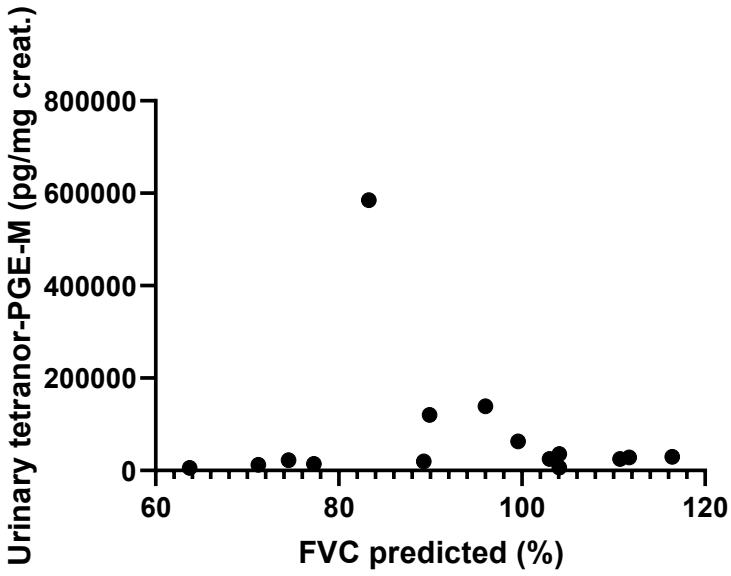

Figure 4.8. Spearman correlation: relationship between urinary tetranor-PGE-M and FVC predicted (%) in children with CF, r=0.35, *p*=0.21.

4.4.1.2. Prostaglandin D2

With regard to PGD_2 metabolites, median (IQR) urinary 13,14-dihydro-15-keto-D2 for CF vs controls were 154 (38 – 263) pg/mg creat vs 51 (28 – 121) pg/mg (*p*=0.07, figure 4.9).

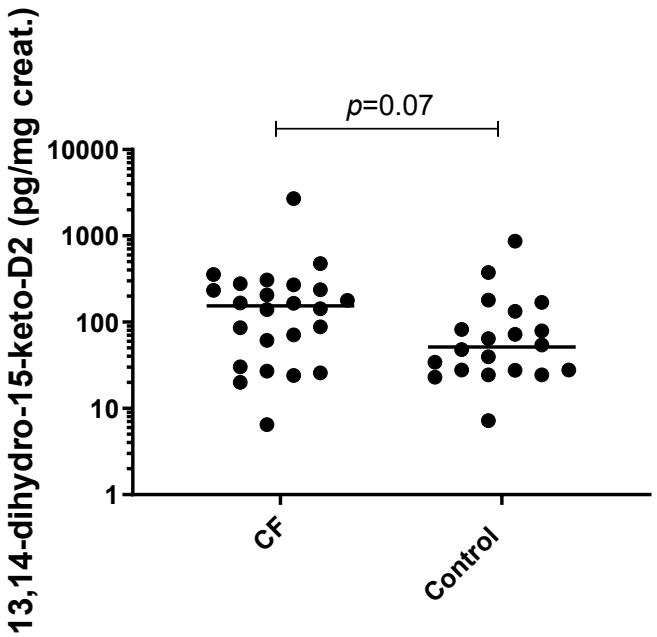

Figure 4.9. Urinary metabolite of prostaglandin D2: 13,14-dihydro-15-keto-D2 for CF and control groups, presented in log scale. Comparison by Mann-Whitney test. Bars represent median.

Median (IQR) urinary 13,14-dihydro-15-keto-tetranor-PGD2 for CF vs controls were 680 (443 – 889) pg/mg creat vs 233 (170 – 405) pg/mg creat (p<0.0001, figure 4.10).

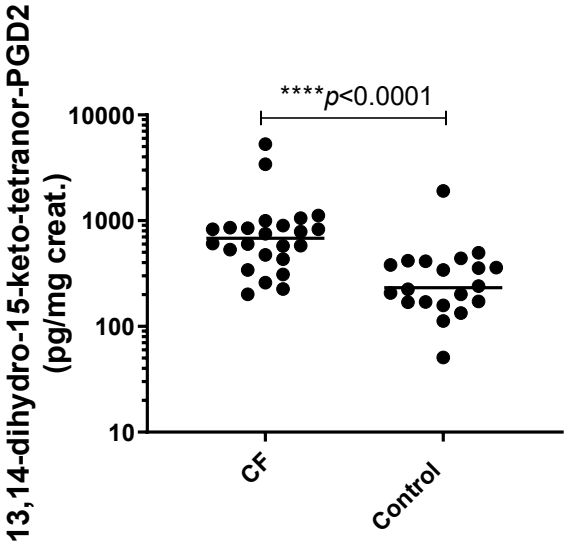

Figure 4.10. Urinary metabolite of prostaglandin D2: 13,14-dihydro-15-keto-tetranor-PGD2 for CF and control groups, presented in log scale. Comparison by Mann-Whitney test. Bars represent median.

Median (IQR) urinary tetranor-PGD-M, the main metabolite of PGD_2, were 8003 (3817 – 12173) pg/mg creat vs 3219 (2567 – 5150) pg/mg creat (p<0.001, figure 4.11)

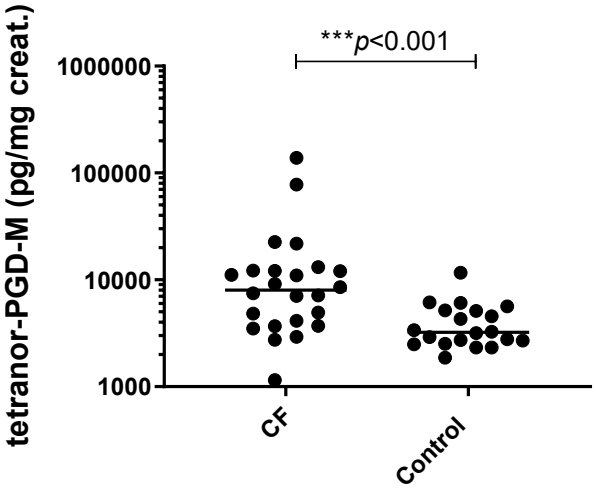

Figure 4.11. Urinary metabolite of prostaglandin D2: tetranor-PGD-M for CF and control groups, presented in log scale. Comparison by Mann-Whitney test. Bars represent median.

Median (IQR) urinary 9a,11b-PGF2 were 868 (572 – 1453) pg/mg creat vs 583 (469 – 787) pg/mg creat (*p*=0.008, figure 4.12).

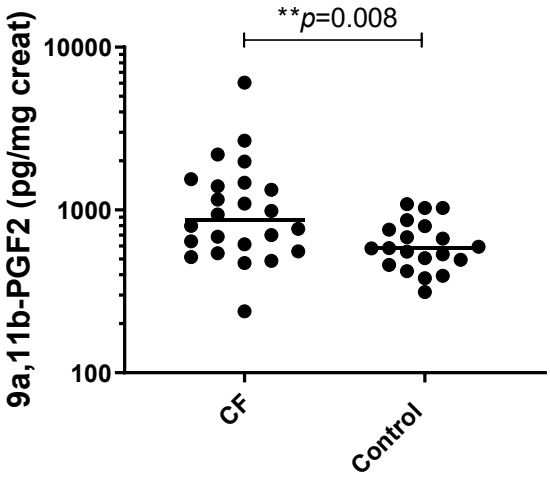

Figure 4.12. Urinary metabolite of prostaglandin D2: 9a,11b-PGF2 for CF and control groups, presented in log scale. Comparison by Mann-Whitney test. Bars represent median.

4.4.1.3. Other eicosanoids

The median (IQR) of PGJ_2 metabolite, urinary 15-doexy-delta12,14-PGJ2, in CF and controls were 4.3 (2.3 – 13.3) vs 2.9 (1.6 – 3.3) pg/mg creat. (*p*=0.02, figure 4.13).

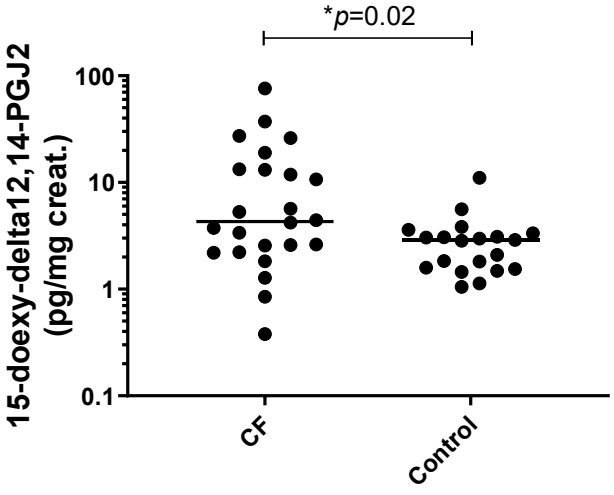

Figure 4.13. Urinary metabolites of prostaglandin J2: 15-doexy-delta12,14-PGJ2, of CF and control groups, presented in log scale. Comparison by Mann-Whitney test. Bars represent median.

Urinary leukotriene E4 (LTE4) levels were similar in both CF and control groups, with median (IQR) being 109 (51 – 181) pg/mg creat and 89 (50 – 112) pg/mg creat respectively (p=0.14, figure 4.14).

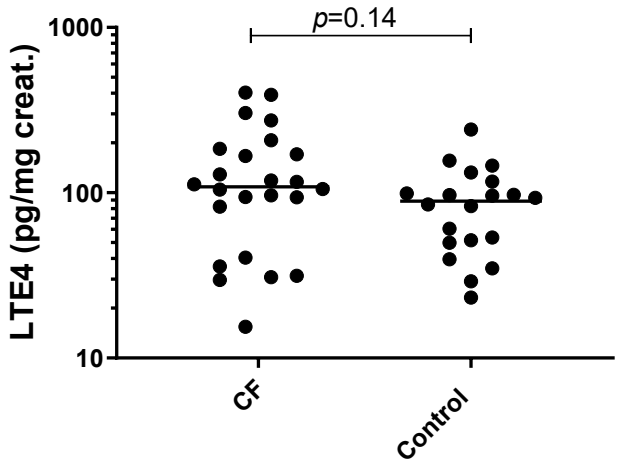

Figure 4.14. Urinary leukotriene E4 (LTE4) of CF and control groups, presented in log scale. Comparison by Mann Whitney test. Bars represent median.

Collectively, these findings show that the urinary metabolites for PGE_2, PGD_2, and PGJ_2 are higher in the CF group compared to healthy controls, supporting the hypothesis that PGs production is increased in CF.

4.4.2. Direct measurement of prostanoid production – sputum supernatant profiles

Supernatant from induced sputum from both CF (n=7) and control (n=9) groups showed that the CF group had higher levels of PGE_2. Median (IQR) were 989 (723 – 1118) pg/mg protein vs 690 (565 – 800) pg/mg protein, *p*=0.04 (figure 4.15).

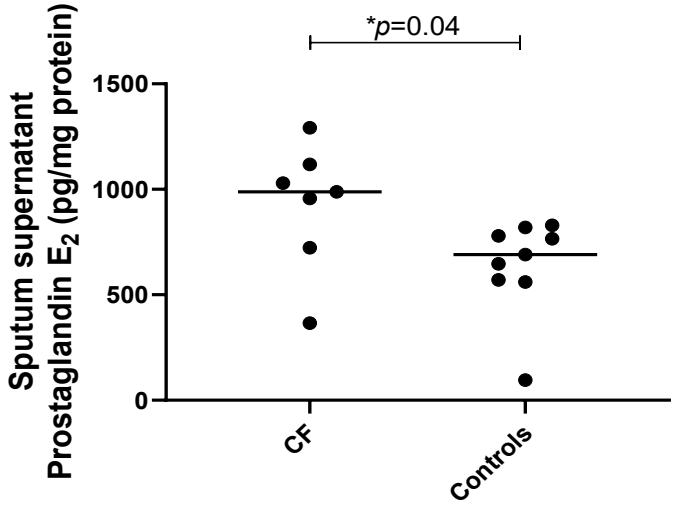

Figure 4.15. Sputum supernatant PGE_2 levels of CF and control groups. Comparison by Mann-Whitney test. Bars represent median.

4.5. Effects of prostaglandin on *in vitro* alveolar macrophage function

As discussed in section 1.7.2.1, and supported by the studies summarised in appendix 2, prostaglandin E2 is known to inhibit macrophage function. This study used enriched primary human AMs to replicate this effect for the first time. Indeed, prostaglandin E2 was shown to inhibit primary AM phagocytosis of diesel exhaust particles *in vitro*.

Following exposure of AMs isolated from CF participants (n=6) to DEP in the absence and presence of PGE_2, the mean difference ± SE between control cultures and those exposed to DEP alone was 12.49 ± 2.79 μm^2, p=0.01; and the mean difference ± SE between cultures exposed to DEP alone and those exposed to DEP and PGE_2 was -5.81 ± 2.32 μm^2, p=0.05 (figure 4.16).

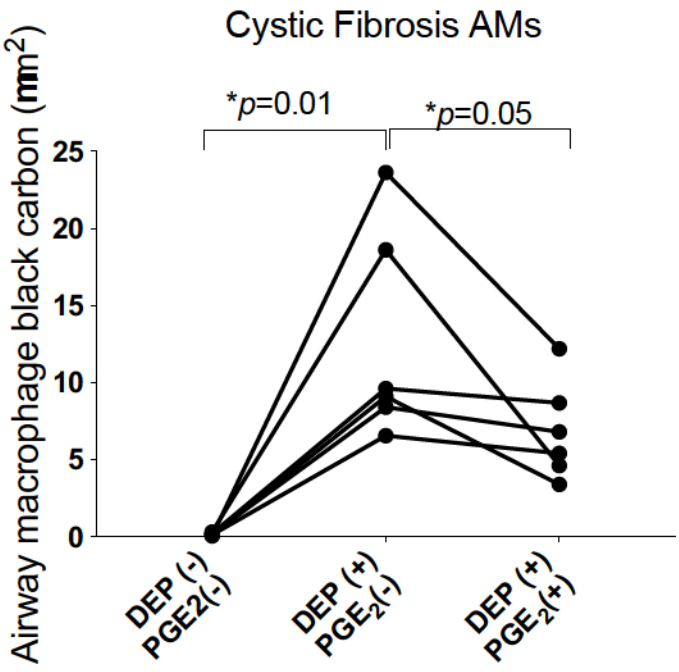

Figure 4.16. Using AMs from CF participants: comparison of alveolar macrophage black carbon in cultures: control (no DEP or PGE_2) vs DEP vs DEP and PGE_2. Comparison by one-way ANOVA.

Using responder AMs isolated from healthy controls (n=14), cells were exposed to DEP in the absence and presence of PGE_2. The mean difference ± SE between control cultures and those exposed to DEP alone was 9.89 ± 1.17 μm^2, $p<0.0001$; while the mean difference ± SE between cultures exposed to DEP alone and those exposed to DEP and PGE_2 was -3.88 ± 0.74 μm^2, $p<0.001$ (figure 4.17).

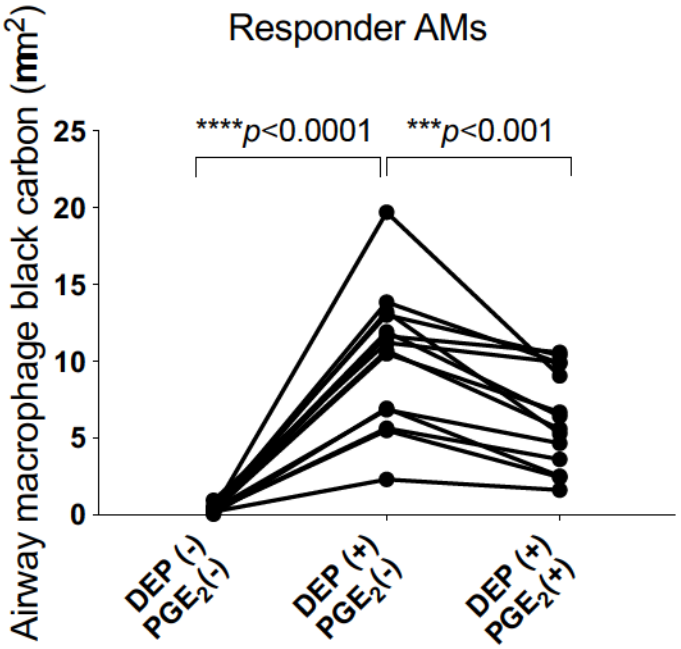

Figure 4.17. Using responder AMs from healthy controls: comparison of alveolar macrophage black carbon in cultures: control (no DEP or PGE_2) vs DEP vs DEP and PGE_2. Comparison by one-way ANOVA.

4.5.1. Reversal of prostaglandin effects on alveolar macrophage function with EP2 antagonist

Using responder AMs from healthy controls (n=5), 75 μM of EP2-receptor antagonist was used to block the action of PGE_2 in the cell-DEP-PGE_2 culture. Firstly, it was demonstrated that EP2 antagonist alone did not affect AM phagocytosis, the mean difference ± SEM in AMBC between cell-DEP and cell-DEP-EP2 cultures was 0.42 ± 0.69 μm^2, p=0.57 (figure 4.18).

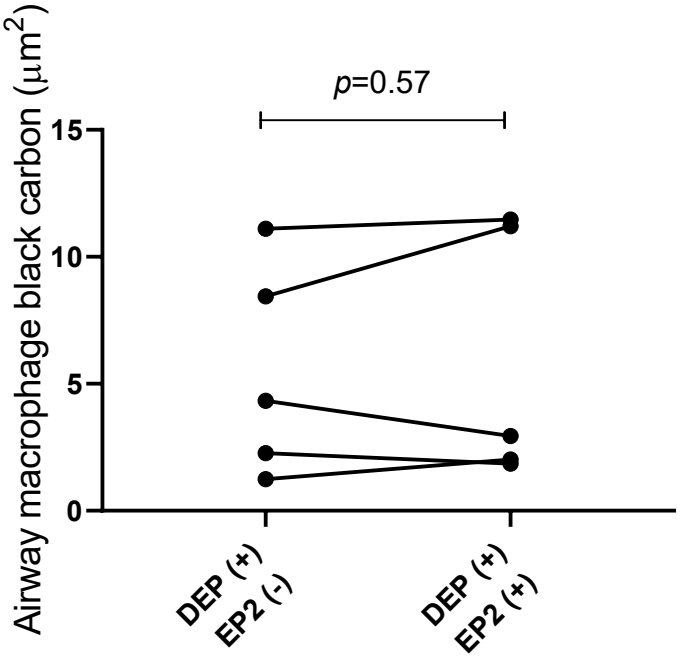

Figure 4.18. Alveolar macrophage black carbon of cell-DEP cultures with and without EP2 antagonist, using responder alveolar macrophages from healthy controls. Comparison by paired t-test.

As shown in figure 4.19, the EP2 antagonist reversed the action of PGE_2, resulting in a mean difference ± SE in AMBC between cultures treated with DEP alone and those treated with DEP and PGE_2 of -2.62 ± 0.74 μm^2 (p=0.05); while mean difference ± SE in AMBC between cultures treated with DEP and PGE_2 and those treated with DEP, PGE_2 and EP2 was 1.77 ± 0.30 μm^2 (p=0.01). There was no significant difference in AMBC between cultures treated with DEP alone and those treated with DEP, PGE_2 and EP2 (mean difference 0.86 ± 0.81 μm^2, p=0.59).

These results once again demonstrate the inhibitory effect of PGE_2 on AM phagocytosis, as seen in figures 4.16 and 4.17. They also prove that blockade of EP2 receptors can prohibit PGE_2 activity on AM phagocytosis.

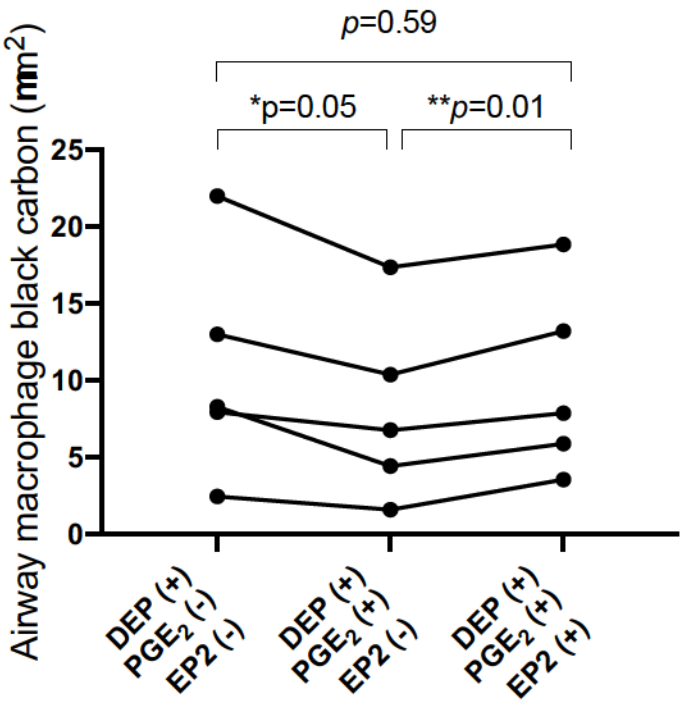

Figure 4.19. Using responder alveolar macrophages from healthy controls: alveolar macrophage black carbon of cell-DEP cultures treated with or without PGE_2 and/or EP2 antagonist. Comparison by one-way ANOVA.

4.6. Effects of CF airway secretions on alveolar macrophage function

In order to further investigate if PGE_2 in CF airway secretions is indeed the cause of impaired AM phagocytosis, sputum supernatant from CF participants was added to the cell-DEP culture to assess responder AM function.

CF sputum supernatant (i.e. diluted cell-free airway secretion) demonstrated inhibitory effects on diesel exhaust particles phagocytosis by responder AMs isolated from healthy controls (n=14). The median of differences (IQR) in AMBC between cell-DEP cultures untreated and treated with CF supernatant was -3.82 (-6.69 to -1.08) μm^2, $p<0.001$ (figure 4.20).

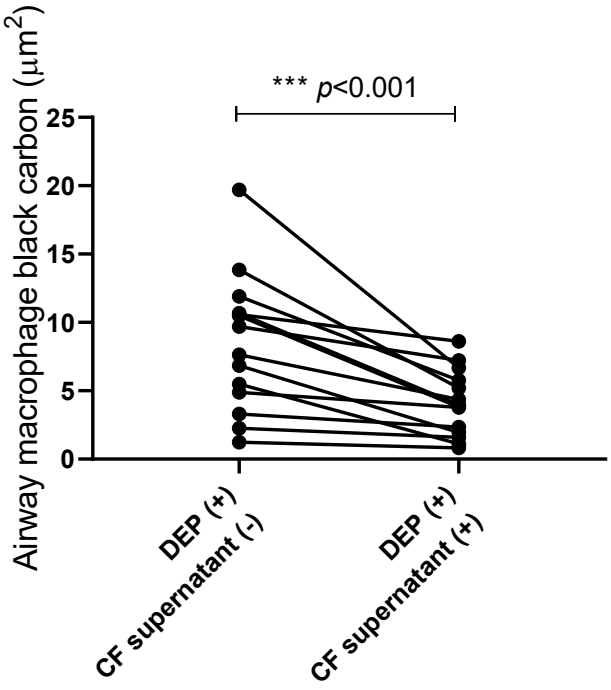

Figure 4.20. Comparison of alveolar macrophage black carbon in cell-DEP cultures untreated (-) and treated (+) with CF supernatant, using responder alveolar macrophages from healthy controls. Comparison by Wilcoxon test.

4.6.1. Reversal of CF airway secretions' effects on alveolar macrophage function with EP2 antagonist

75μM of EP2-receptor antagonist was used to block the potential action of PGE_2 in the CF supernatant. EP2 antagonist was able to restore responder AM phagocytosis of diesel exhaust particles. The mean difference ± SE in AMBC between cultures exposed to DEP in the absence and presence of CF supernatant was -2.78 ± 0.93 μm^2, p=0.04; the mean difference ± SE in AMBC between cultures exposed to DEP and treated with CF supernatant, in the absence and presence of EP2 antagonist, was 1.66 ± 0.53 μm^2, p=0.04. There was no significant difference in AMBC between exposed cultures in the absence of CF supernatant and EP2 antagonist, and those in the presence of CF supernatant and EP2 antagonist (mean difference ± SE of 1.12 ±1.06 μm^2, p=0.57), figure 4.21.

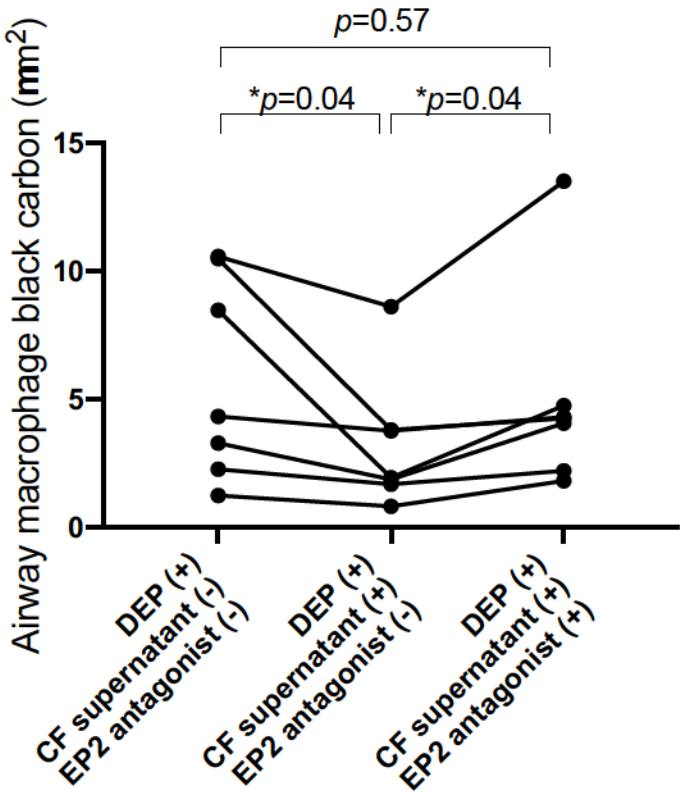

Figure 4.21. Using responder alveolar macrophages from healthy controls: alveolar macrophage black carbon in cell-DEP cultures treated with or without CF supernatant and/or EP2 antagonist. Comparison by one-way ANOVA.

4.7. Effects of Cyclooxygenase inhibitor on Prostanoid Profiles

Urinary PGE_2 metabolite levels were measured in a random selection of children with cystic fibrosis before and after a 3-day course of over-the-counter-dose of Ibuprofen. Their lung function (FEV_1 predicted) at the beginning of Ibuprofen course is listed in table 4.1.

Patient	FEV1 predicted (%)
1	59.1
2	76.5
3	75.6
4	87.5
5	73.1
6	101
7	70.4

Table 4.1. Lung function (forced expiratory volume in 1 second, FEV_1 % predicted) of CF participants at the beginning of the 3-day Ibuprofen course.

For urinary 13,14-dihydro-15-keto-PGE_2, the median of difference (IQR) between pre- and post- Ibuprofen was -2286 (-4500 to -351) pg/mg creat. (p=0.03, n=6) (figure 4.22).

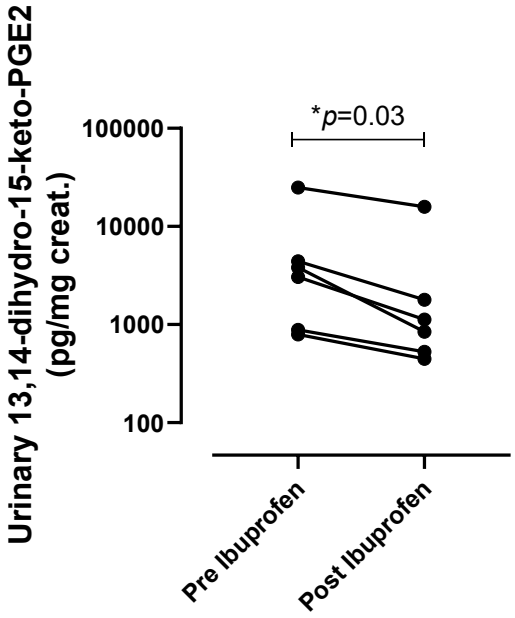

Figure 4.22. Urinary 13,14-dihydro-15-keto-PGE_2 of children with cystic fibrosis, before and after a 3-day course of standard dose Ibuprofen, presented in log scale. Comparison by Wilcoxon test.

For urinary 13,14-dihydro-15-keto-tetranor PGE_2, the median of difference between pre- and post- Ibuprofen was -176 (-458 to -75) pg/mg creat. (p=0.02, n=7) (figure 4.23).

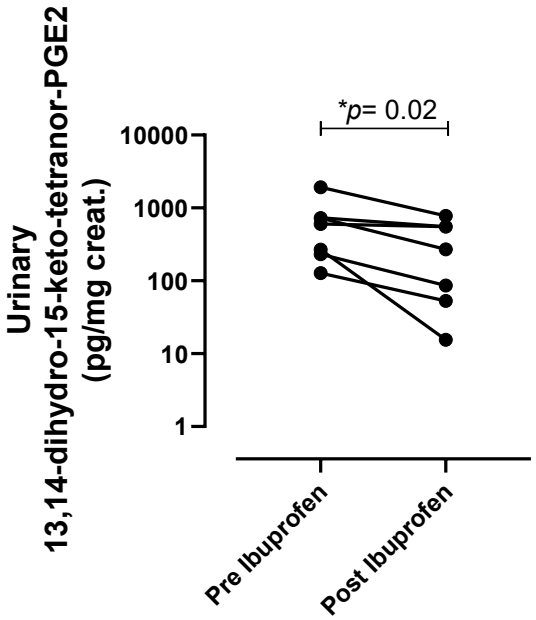

Figure 4.23. Urinary 13,14-dihydro-15-keto-tetranor PGE_2 of children with cystic fibrosis, before and after a 3-day course of standard dose Ibuprofen, presented in log scale. Comparison by Wilcoxon test.

For urinary tetranor PGE-M (main metabolite of PGE_2), the median of difference (IQR) between pre- and post- Ibuprofen was -70989 (-107711 to -281) pg/mg creat. (p=0.04, n=7) (figure 4.24).

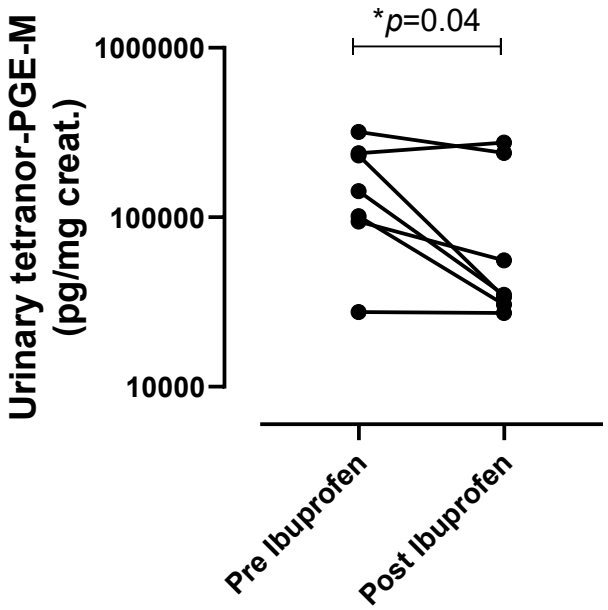

Figure 4.24. Urinary tetranor PGE-M of children with cystic fibrosis, before and after a 3-day course of standard dose Ibuprofen, presented in log scale. Comparison by Wilcoxon test.

These results suggest that a short 3-day course of standard dose Ibuprofen can lower urinary metabolites of PGE_2 in children with CF, suggesting systemic reduction in PGE_2 production.

In this work, sputum concentrations of PGE_2 were not measured pre- and post-Ibuprofen course due to time constraints. AMBC was also not determined pre- and post- Ibuprofen course because the turnover time for AMs is approximately 3 months, therefore participants will have to be on a longer course of Ibuprofen in order to demonstrate any changes on AMBC loading.

When the urinary PGE_2 metabolite levels of children with CF after a 3-day course of Ibuprofen were compared with the metabolite levels from healthy controls (baseline levels with no Ibuprofen), Ibuprofen seemed to be able to reduce the CF urinary metabolites to comparable levels as controls for two of the three PGE_2 metabolites measured.

For urinary 13,14-dihydro-15-keto-E2, the median (IQR) for CF (post-Ibuprofen) vs control were 985 (509 – 5301) pg/mg creat. (n=6) vs 928 (478 – 1376) pg/mg creat. (n=20) (*p*=0.61) (figure 4.25).

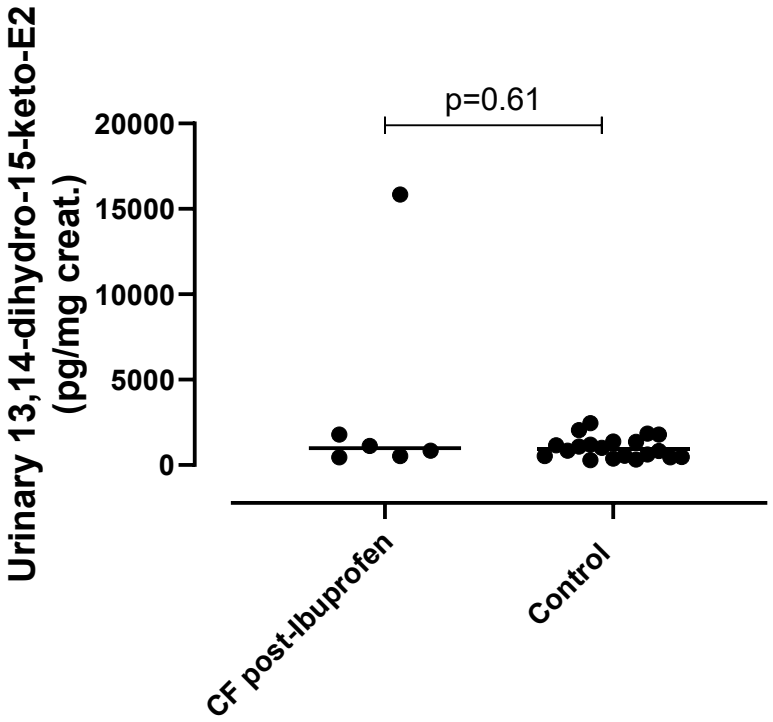

Figure 4.25. Urinary 13,14-dihydro-15-keto-E2 of children with CF after a 3-day course of Ibuprofen and healthy controls. Comparison by Mann-Whitney test. Bars represent median.

For urinary 13,14-dihydro-15-keto-tetranor E2, the median (IQR) for CF (post-Ibuprofen) vs control were 270 (53 – 554) pg/mg creat. (n=7) vs 214 (96 – 323) pg/mg creat (*p*=0.77) (figure 4.26).

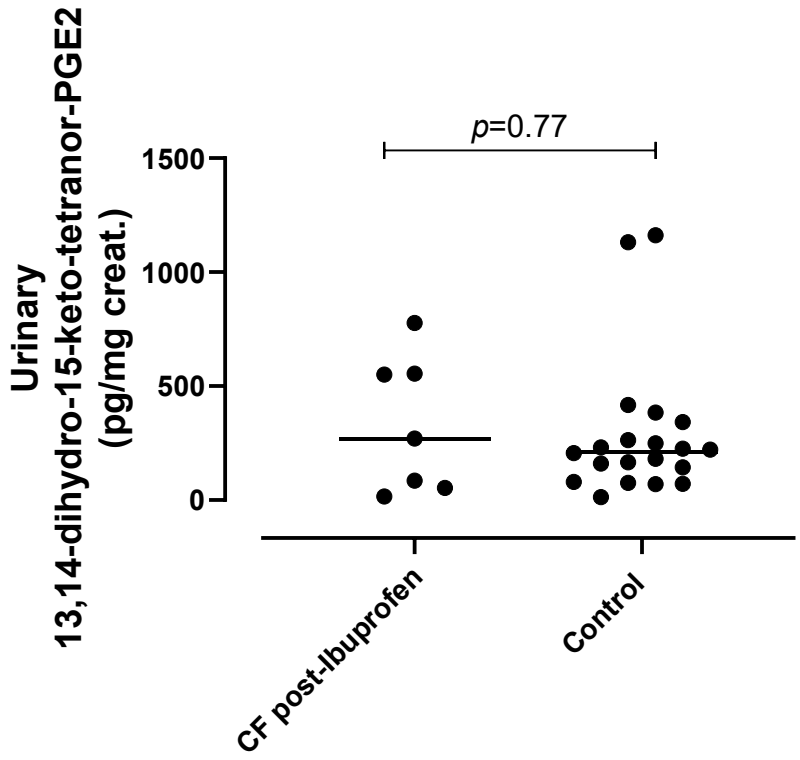

Figure 4.26. Urinary 13,14-dihydro-15-keto-tetranor E2 of children with CF after a 3-day course of Ibuprofen and healthy controls. Comparison by Mann-Whitney test. Bars represent median.

For urinary tetranor PGEM, the median (IQR) for CF (post-Ibuprofen) vs control were 34851 (30579 – 239795) pg/mg creat. (n=7) vs 15873 (10150 – 24733) pg/mg creat. (n=20) (p<0.001) (figure 4.27).

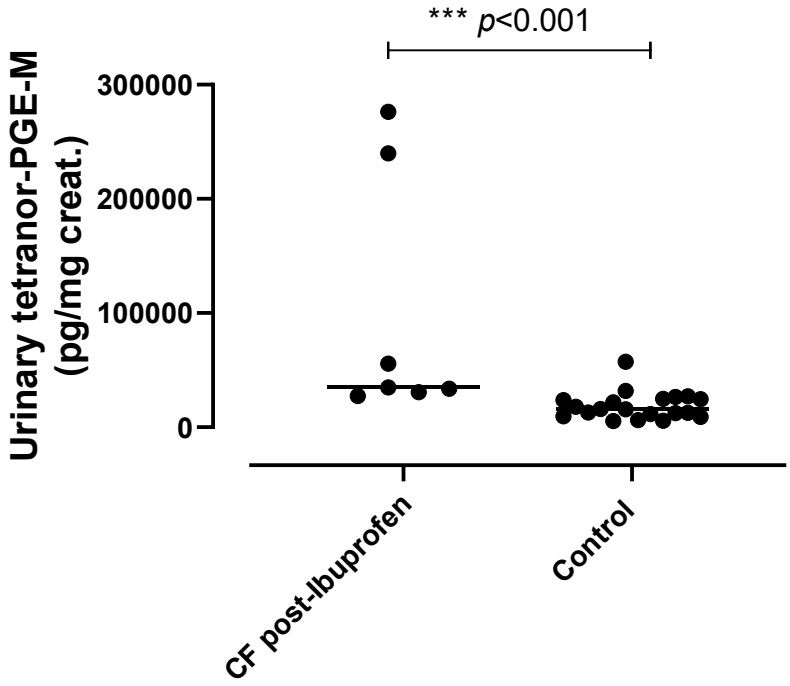

Figure 4.27. Urinary tetranor PGEM of children with CF after a 3-day course of Ibuprofen and healthy controls. Comparison by Mann-Whitney test. Bars represent median.

For urinary tetranor PGEM, the furthest outlier in the CF group corresponds to the participant with the lowest lung function (FEV_1 predicted) at the beginning of the Ibuprofen course (table 4.1).

These results show that, apart from urinary tetranor PGEM, a 3-day course of Ibuprofen was able to reduce urinary 13,14-dihydro-15-keto-E2 and 13,14-dihydro-15-keto-tetranor E2 in the CF group to comparable levels seen in the control group.

4.8. Discussion

The results of the previous chapter suggested that AM phagocytosis impairment in CF is likely to be secondary to inhibitory substances present in CF airway secretions, with PGE_2 suspected to be one of the mediators. As mentioned in section 1.7.2., CF airways are known to contain excessive cytokines and PGs; BAL, sputum, exhaled air and saliva from patients with CF do indeed contain higher levels of PGE_2 [144-148]. Chen *et al*. [137] described CFTR as a regulator of the PGE_2-mediated inflammatory response; that a lack of functional CFTR could lead to heightened inflammatory signalling.

The results from this chapter confirm that PG production is increased in CF, reflected by higher levels of urinary metabolites of PGE_2, PGD_2 and PGJ_2, and supported by the increased COX-2 expression in AMs isolated from the CF group. These findings are in agreement with the upregulation of COX-2 in CF nasal polyps reported by Roca-Ferrer *et al.* [140]. Besides, the higher concentration of PGE_2 in CF sputum supernatant compared to the control group, also supports that PGE_2 production is amplified in CF.

Aronoff *et al*. [141] described that PGE_2 could inhibit AM phagocytosis in a dose-dependent manner, as demonstrated in the findings from this study. Domingo-Gonzalez *et al*.[183] also reported in an animal model that there was an impaired innate immune response driven by elevated PGE_2 after bone marrow transplant; and treatment with PGE_2 could result in altered bacterial phagocytosis due to changes in the AM scavenger receptor profile. The *in vitro* model in this study demonstrated the inhibitory effect of PGE_2 on responder AM (those extracted from

healthy controls) phagocytosis, which was restored by an EP2 receptor antagonist. Interestingly, cell-free CF sputum supernatant, containing CF airway secretions and mucus, also exerted inhibitory effects on responder AM phagocytic function. Since PGE_2 was the suspected inhibitory factor in CF airway secretions, an EP2 receptor antagonist was used to show any potential reversal of AM phagocytosis inhibition by CF sputum supernatant. Indeed, the EP2 antagonist restored responder AM function despite the presence of CF secretions, suggesting PGE_2 does play a role in phagocytic impairment in CF.

Ibuprofen is a non-steroidal anti-inflammatory drug, which acts by inhibiting COX-1 and COX-2. Clinical trials have shown that high-dose ibuprofen can reduce inflammation and slow the disease progression in CF, especially in children [184,185]; however, ibuprofen used at high dose has side effects. A low over-the-counter dose of ibuprofen was used in this work, and a reduction in systemic PG production was demonstrated only after a short 3-day course. Interestingly, for two of the three PGE_2 metabolites examined, Ibuprofen was able to reduce the urinary PG metabolite levels of the CF group to that similar of the control group – although the short course of Ibuprofen did not lower CF urinary tetranor-PGEM to levels comparable to control group, its production was still reduced, whether a longer course would be able to lower its production further is yet to be explored.

Considering the inhibitory effects of PGE_2 on AM function, as demonstrated in this chapter, less systemic PGE_2 production could potentially lead to improved AM phagocytosis, thereby reducing the vulnerability of children with CF to inhaled particulate matter.

Although a short course of Ibuprofen at a low dose may not affect CF disease progression as such, longer term usage may offer potential therapeutic benefits in improving AM function in face of air pollutants.

4.8.1. Strengths

COX-2 expression in AMs from CF and control groups were determined using flow cytometry. Flow cytometry allows for elimination of cell debris and dead cells during analysis. The cell populations presented for flow cytometry were enriched sputum samples consisting mostly of AMs; the cells were further marked with a macrophage marker in order to enhance flow cytometry gating. Prostaglandins were measured both in the urine (a traditional approach, indirect measure of PG production) and sputum supernatant (direct measure of PG production), to confirm that PGs, particularly PGE_2, were indeed present in higher quantities in CF.

4.8.2. Limitations

Monocytes tend to be adhesive, making flow cytometry slow and difficult at times. Vigorous agitation of samples before flow cytometry was therefore required. As before, the *in vitro* assays were limited by the number of AMs available for each experiment due to varying participants' sputum induction techniques, and inevitable cell loss during processing and experimentation. Due to limited number of cells for flow cytometry, any variation in gating strategy could result in drastic changes in median MFI, making gating by a second observer challenging. In order to address this and limit bias, blinded re-analyses were performed by the same researcher (myself) and results from the blinded and unblinded analyses were compared.

The data presented in this chapter are limited in power due to the small number of participants who underwent repeated sputum induction for the PG experiments.

Since CF airway secretions are thick, the reduction in responder AM phagocytic ability when exposed to CF sputum supernatant could be secondary to its viscosity, impeding AM access to DEP. However, the successful reversal of phagocytic function using an EP2 antagonist suggested such functional impairment was related to PGE_2.

Ibuprofen was used at a standard over-the-counter course for 3 days. While this showed a reduction in PG metabolites in the urine, whether this would in fact improve AM function remains unclear. With the average clearance half-life of AMBC reported to be 53 to 116 days by Bai *et al*. [104], children will have to take daily Ibuprofen for at least 3 months (the "wash-out" period) before any changes in AMBC can be seen. This was not done in this study because of time limitation, but would certainly be an interesting area to explore in the future.

4.8.3. Summary

The results from this chapter confirm the increased production of PGs in CF, secondary to increased expression of COX-2 in AMs in this group. Using responder AMs from healthy controls, PGE_2 was shown to have an inhibitory effect on AM phagocytosis *in vitro*, which was successfully restored by an EP2 antagonist. Interestingly, a similar phagocytic-inhibitory effect was seen with cell-free sputum supernatant from children with CF, this was also restored by an EP2 antagonist.

This strongly suggests that PGE_2 is indeed the mediator responsible for impaired AM phagocytosis *in vivo* in CF. So far, this thesis has demonstrated that AM phagocytosis of BC is impaired in CF, likely secondary to increased levels of PGs in the CF airway. In the next chapter, the consequence of such AM functional impairment will be explored.

Chapter 5: Modelling the effects of impaired alveolar macrophage phagocytosis caused by prostaglandin E2

5. Modelling the effects of impaired alveolar macrophage phagocytosis caused by prostaglandin E2

5.1. Background

With reduced AM functional capacity, non-phagocytosed inhaled carbonaceous particulate matter may remain in the airway and exert its adverse effects on other airway cells such as epithelial cells. These effects are typically pro-inflammatory. Indeed, as discussed in section 1.4.2, upon exposure to insults such as diesel exhaust particles, the airway epithelium contributes to inflammatory cytokine release, inflammatory cell differentiation and activation – a phenomenon that is already intrinsically prominent in the CF airway even without the presence of air pollution, as mentioned in section 1.7.2. Chronic airway inflammation is the driver of structural lung damage and lung function decline in CF, which could be worsened by exposure to air pollution.

In sections 1.2.1.1. and 1.4.3., the effects of air pollutants on epithelial cells were explored. Other than mediating inflammation, DEP and NO_2 can also affect epithelial ciliary movements, alter cell integrity and increase cell permeability; increasing the chance of particle translocation by passive diffusion. In addition, uptake of nanoparticles by epithelial cells can occur through endocytosis.

5.2. Aims

In this chapter, the hypothesis tested is:

- In the absence of functional alveolar macrophages, particulate matter invades other airway cells such as epithelial cells, inducing pro-inflammatory cascades and the release of cytokines such as IL-8.

The consequences of non-phagocytosed diesel exhaust particles remaining in the airway are explored. The potential for diesel exhaust particles to invade epithelial cells is investigated, using an epithelium-macrophage *in vitro* model (figure 5.1), containing A549 adenocarcinomic human alveolar basal epithelial cells and responder alveolar macrophages from healthy participants. The concept of how functional alveolar macrophages are important to protect epithelial cells and the lungs, is demonstrated using this model followed by image analysis. Cytokine (IL-8) release from A549 epithelial cells when exposed to DEP is also examined.

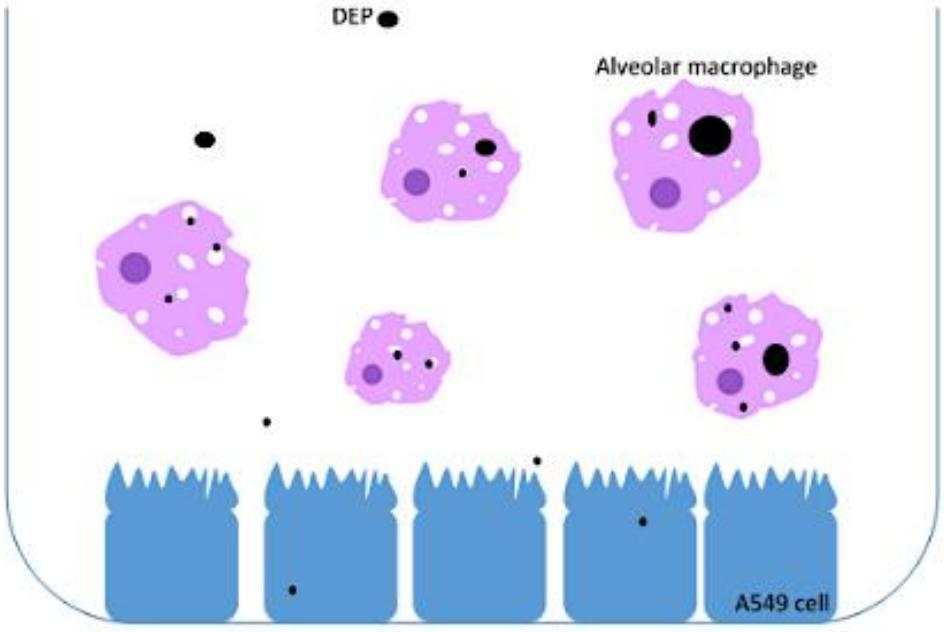

Figure 5.1. Epithelium-macrophage *in vitro* model: A549 epithelial cells adhered to the well overnight, followed by addition of primary responder alveolar macrophages and DEP. Functional alveolar macrophages will phagocytose DEP and protect against invasion of epithelial cells.

5.3. Epithelial cell model

5.3.1. Diesel exhaust particle invasion into epithelial cells

In the absence of alveolar macrophages, diesel exhaust particles were able to invade A549 epithelial cells during the exposure period (figure 5.2).

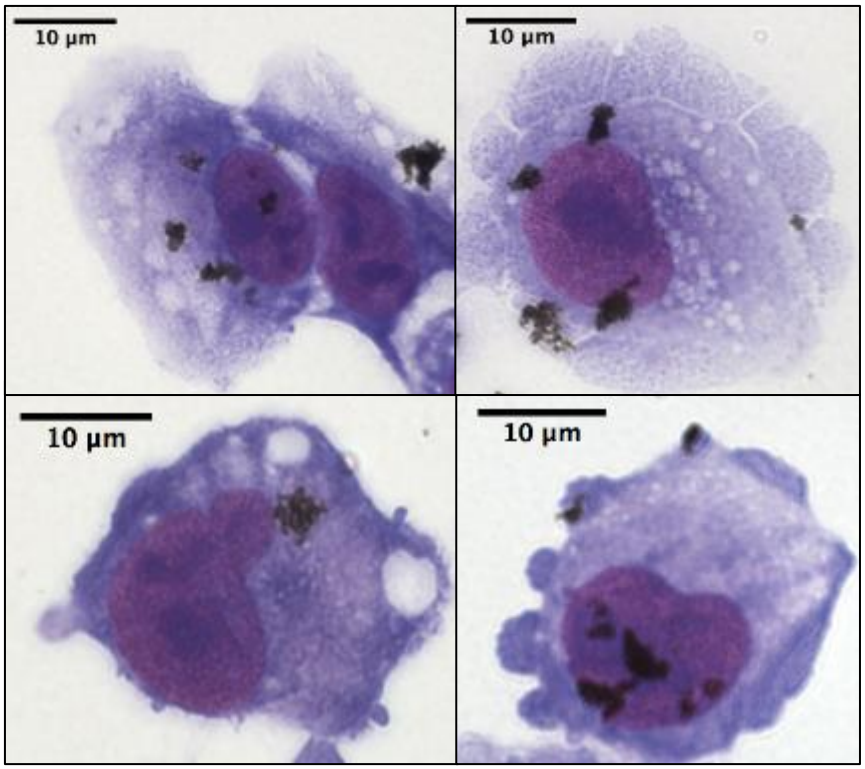

Figure 5.2. Light microscopy of A549 epithelial cells following exposure to diesel exhaust particles, showing particle invasion into the cells.

5.3.2. Protective role of airway macrophages

Compared with pure epithelial cultures, the area of epithelial black carbon was significantly reduced in the presence of responder AMs. Examining 50 randomly selected epithelial cells per culture, mean ± SEM epithelial BC were 12.80 ± 2.45 μm^2 vs 3.19 ± 1.17 μm^2 for control cultures (no AMs) and cultures with AMs respectively, with a median of difference (IQR) of -9.50 (-13.10 to -4.94) μm^2 (n=6, *p*=0.03, figure 5.3a), and a mean ± SEM percentage reduction of -75.12 ± 8.85%. Digital images showed that the majority of carbon in mixed epithelial-macrophage cultures was phagocytosed by adjacent AMs (figure 5.3b).

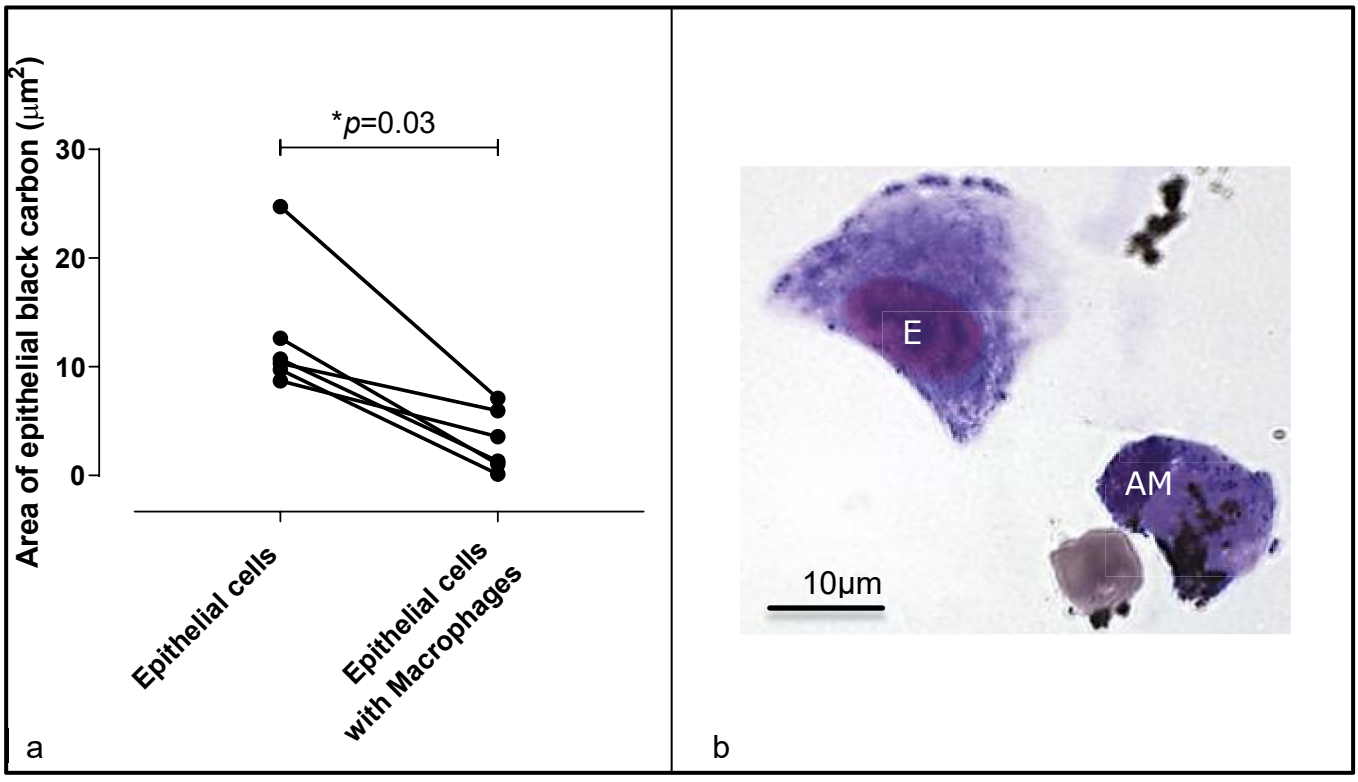

Figure 5.3. (a) Area of black carbon in A549 epithelial cells with and without alveolar macrophages. Comparison by Wilcoxon test. (b) A549 epithelial cell (E) and a neighbouring primary alveolar macrophage (AM) which has phagocytosed black carbon, protecting the epithelial cell from black carbon invasion.

5.3.3. Effects of prostaglandin E2 on the function of alveolar macrophages

The results in section 4.5. showed the inhibitory effect of PGE_2 on AM function. Black carbon associated with A549 cells were increased in the epithelial-macrophage cultures with PGE_2, compared to those without PGE_2. The median of difference (IQR) was 0.64 (0.36 – 6.59) μm^2 (n=6, *p*=0.03, figure 5.4).

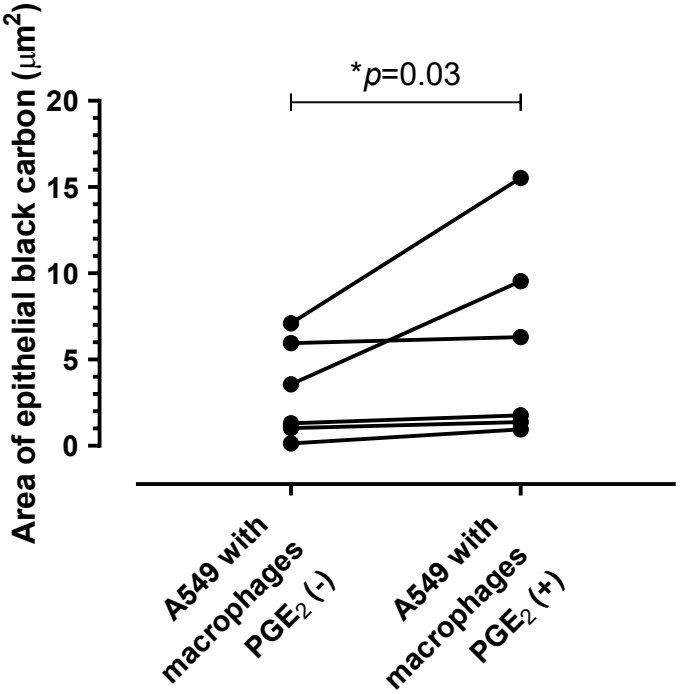

Figure 5.4. Area of black carbon in A549 epithelial cells in the presence of alveolar macrophages, with and without PGE2. Comparison by Wilcoxon test.

5.4. Cytokine release from epithelial cells following diesel exhaust particles exposure

Interleukin 8 (IL-8) levels between cultures of i) controls (A549 cells only); and ii) A549 cells and 10 µg/ml of DEP, were compared. ELISA standard curve shown in figure 5.5.

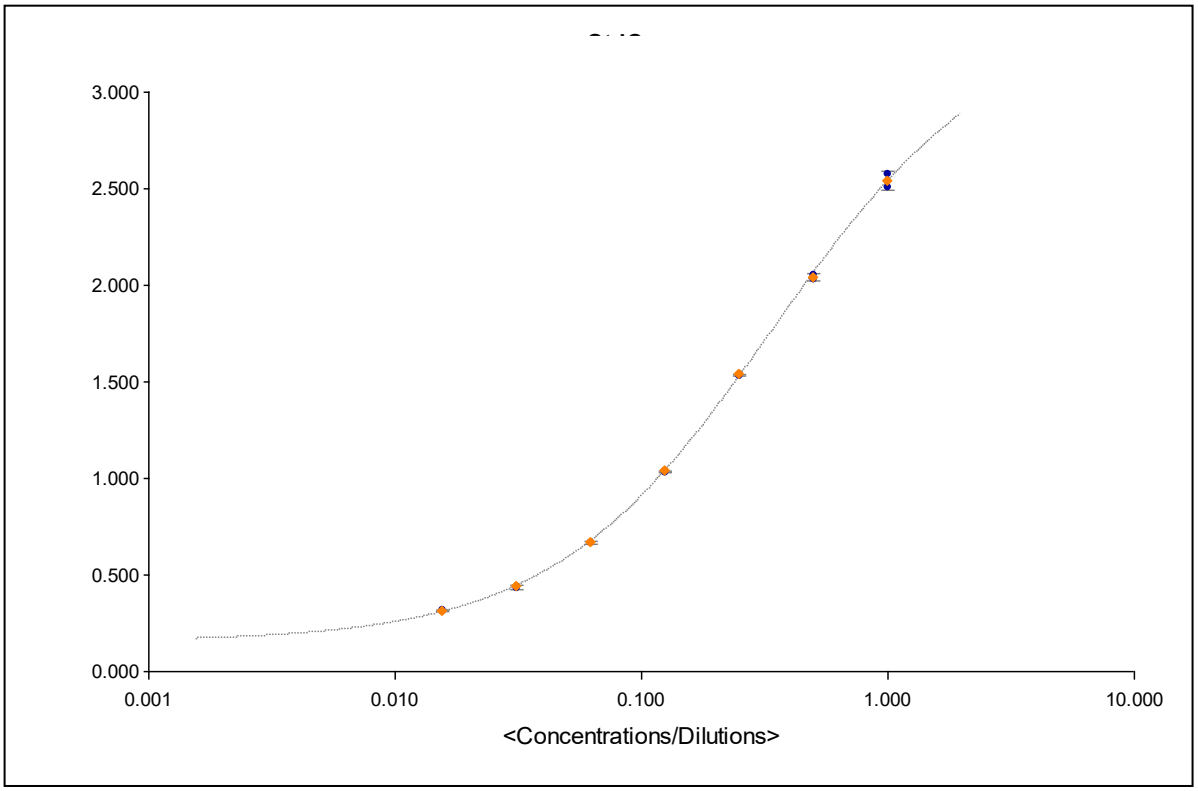

Figure 5.5. Standard curve created using a curve-fitting software, subtracting the background absorbance. $R^2 = 1$.

The median of difference (IQR) IL-8 levels for A549 only vs A549 exposure to DEP was 49.79 (24.07 – 96.10) pg/ml (n=9, *p*=0.004) (figure 5.6a). Similar trend was seen when data were transformed into fold change. The median (IQR) fold change between control cultures of A549 alone and cultures treated with DEP was 2.16 (1.27 – 2.97), n=9, *p*<0.0001 (figure 5.6b).

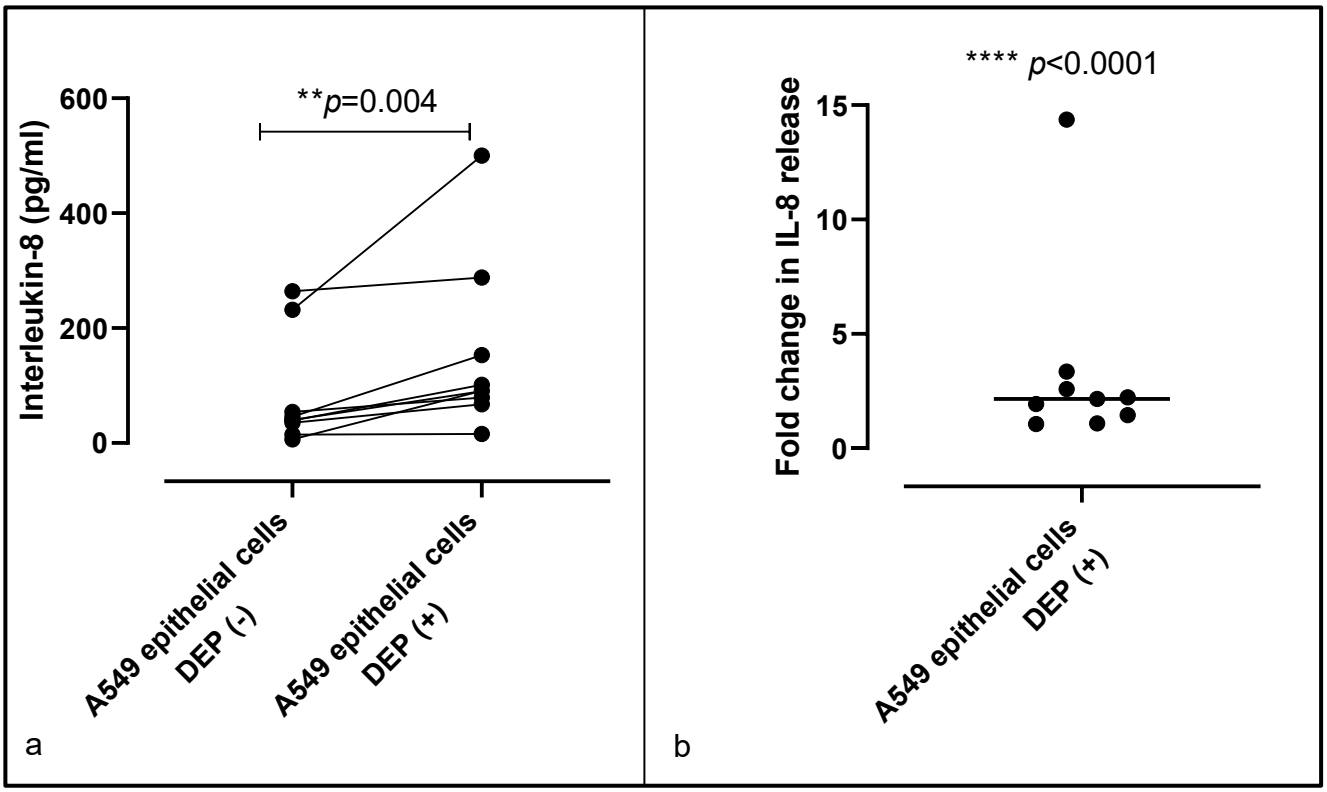

Figure 5.6. (a) Interleukin 8 (IL-8) levels: cultures of A549 epithelial cells with and without 10 µg/ml DEP. Comparison by Wilcoxon test. (b) Fold change in IL-8 release in epithelial cell cultures exposed to DEP, compared to those unexposed. Comparison by Mann-Whitney test. Bar represents median.

5.5. Discussion

With impaired AM phagocytosis, inhaled carbonaceous particles may remain in the airway and exert their detrimental effects on epithelial cells. As discussed in section 1.2.1, the airway epithelium is an important part of the immune defence against foreign insults. It acts as a physical barrier, and is a medium for ciliary action and mucociliary clearance. Its role in inflammatory and antimicrobial activities is by virtue of the production of cytokines. Rezaee *et al*. [186] suggested that CFTR may be involved in regulating apical membrane proteins, and may therefore have a role in epithelial barrier function – i.e. defective CFTR increases epithelial permeability, thereby increasing the CF host's susceptibility to invading pathogens and particles. Previous research indicated that DEP not only increases cytokine production by human bronchial epithelial cells, but also reduces the cytoskeletal stiffness and cell-matrix adhesion molecule, allowing for epithelial cells detachment [187].

As demonstrated by Stearns *et al.* [87], nanoparticles could adhere to and invade epithelial cells, altering their permeability and integrity. Indeed, the results from this chapter have shown that diesel exhaust particles can invade epithelial cells. This is particularly problematic for individuals with impaired macrophage function such as children with CF. The protective role of alveolar macrophages was demonstrated in the epithelium-macrophage *in vitro* model, where AM phagocytosis of DEP resulted in less epithelial cell invasion. The addition of PGE_2 to the epithelium-macrophage model was an attempt to mimic the CF airway to an extent, the subsequent increase in DEP invasion of epithelial cells demonstrated the potential consequence of non-phagocytosed PM in the CF airways.

It is known that the CF airway is overwhelmed by inflammation caused not only by recurrent infections, but also as part of the disease pathogenesis. CFTR defects can cause abnormal signalling in epithelial cells; while accumulation of defective CFTR can trigger apoptosis and dysregulated immune function, resulting in amplified but ineffective airway inflammation [188]. In CF, reduced airway surface liquid and impaired mucociliary function encourage bacterial growth, leading to neutrophil-driven inflammatory responses. Many studies have found increased levels of IL-8, a neutrophil chemokine, in the CF airway [189]. For example, Sagel *et al*. [190] reported increased neutrophil counts, IL-8 levels, and neutrophil elastase activity in sputum from children with CF compared to healthy controls. Furthermore, IL-8 can also be used as a biomarker for disease severity in CF, as Mayer-Hamblett *et al.* [191] found a negative correlation between IL-8 and lung function.

The ELISA results in this chapter showed that IL-8 release by A549 epithelial cells was increased in the presence of DEP, supporting the hypothesis that epithelial inflammation is aggravated by DEP exposure, thereby potentially worsening disease progression in CF, where AM function is impaired.

Of note, Massengale *et al.* [192] proposed that IL-8 release by CF airway epithelial cells was defective, and may contribute to bacterial colonisation in the airway – they found that IL-8 secretion and IL-8 mRNA were lower in CF epithelial cell line compared to normal epithelial cell line, or CF phenotypically corrected cell line. This was in contrast to other publications which stated that IL-8 secretions were higher in CF cell line compared to CF corrected cell line [193]; the deduction was that this was due to differences in CF genotype or CFTR expression.

5.5.1. Strengths

The lower airway was modelled using A549 epithelial cells and primary AMs from participants. The addition of PGE_2, though at a higher than physiological concentration, mimicked the CF airway to a certain extent. This model demonstrated the invasion of DEP into epithelial cells without the protection from functional AM, along with subsequent inflammatory cytokines release. AMs used were enriched, with saliva and mucus removed – since saliva contains lower levels of IL-8 compared to sputum [190], the enrichment process eliminated this potential confounder.

5.5.2. Limitations

A549 cell lines were used as opposed to primary epithelial cells. The phenotype, architecture, and properties of the cell line may not be truly representative of its primary counterpart. Apart from the limited and variable number of AMs available from each participant, the lack of other inflammatory cells and mucus mean that the epithelium-macrophage model is not an accurate representation of the airway, which is a lot more complex in reality, with many other inflammatory cells and cytokines being activated and interacting with each other – these were not addressed by the model in this work. The results for the epithelium-macrophage assays were obtained from a small number of children, the conclusions drawn are therefore limited by the risk of type 1 and type 2 errors.

5.5.3. Summary

The results from this chapter demonstrate the protective role of functional AMs on epithelial cells, and that non-phagocytosed carbonaceous PM can indeed invade

epithelial cells. As highlighted in previous chapters, *in vivo* function of AMs in CF appears to be impaired, rendering patients more susceptible to the effects of non-phagocytosed PM within the airway. The increase in IL-8 release when A549 epithelial cells were exposed to DEP, supports the hypothesis that non-phagocytosed PM can induce epithelial cytokine release. After demonstrating the potential for non-phagocytosed particles to invade airway epithelial cells, the next chapter will look into potential translocation of non-phagocytosed ultrafine particles, invading cells and organs beyond the respiratory system.

Chapter 6: Translocation of air pollution particles

6. Translocation of air pollution particles

6.1. Background

Having demonstrated that non-phagocytosed ultrafine particulate matter may be taken up by airway epithelial cells, leading to local inflammation, this chapter will explore the potential for any residual inhaled ultrafine or nano particles to translocate across tissue barrier and travel through the circulation to distant organs.

The placenta is a temporary organ formed during pregnancy, and is naturally expelled following childbirth. Therefore, not only can the placenta be used as a proxy for distant organs without the need for surgery or autopsy, any evidence of particle translocation to the placenta may also explain how placental function and fetal development are affected by air pollution.

It is known that apart from the lungs, air pollution can also affect other organs, and that maternal exposure to air pollution can result in adverse birth outcomes such as low birth weight and preterm delivery. While the mechanisms of how pollutants affect distant organs and fetal development are undoubtedly manifold, one of the putative explanations is translocation of pollutants via the circulation, allowing them to exert their toxic effects directly on other organs such as the placenta and fetus.

As discussed in section 1.4.4, ultrafine and nano particles are able to enter the circulation in animal models, whereas *ex vivo* models have shown the possibility of nanoparticles crossing the placental barrier. In order to seek evidence of extra-pulmonary particles in the body, microscopy can be used to visualise any particles phagocytosed by local macrophages.

6.2. Aims

In this chapter, the hypothesis tested is:

- Carbonaceous particulate matter is present in placental tissues, having translocated from the maternal lung after by-passing the maternal first line of respiratory defence.

The possibility of particle translocation from the lung to distant organs is explored. Personal air pollution exposure of the recruited pregnant women were modelled using their home addresses. Evidence of black carbon in placental macrophages was sought using light and electron microscopy.

To investigate the potential for non-phagocytosed inhaled carbonaceous PM to translocate from the lung to distant organs via the systemic circulation, placentas were used. The placenta is a temporary organ which is delivered in the third stage of labour. In a healthy pregnancy followed by the delivery of a healthy term infant, the placenta is usually discarded after delivery. It is therefore a non-invasive way to examine human organs without the need for surgical intervention or autopsy.

6.3. Methods

6.3.1. Ethical approval

The project protocol was reviewed and approved by the Joint Research Management Office for Barts Health NHS Trust and Queen Mary University of London.

Ethical approval to recruit women was granted by the NHS Research Ethics Committees (REC, REC references 17/NW/0092, IRAS project ID 219053) in February 2017, following a full ethical review. Health Research Authority (HRA) approval was obtained in March 2017. See appendix 8 for the latest version of the study protocol.

Three subsequent amendments were approved for the following reasons:

I. To expand the number of participants, and include personal nitrogen dioxide monitoring in participants.
II. To change the timeframe between information sheet dissemination to consent from at least 24 hours to the morning of elective caesarean sections.
III. To extend the study date for another year.

See appendix 11 for all correspondences with the ethics committee.

6.3.2. Participants

Healthy pregnant women were recruited from the Royal London Hospital tertiary maternity unit. They were healthy women who were booked for elective Caesarean sections (C-sections) to deliver a healthy singleton infant. Elective C-section was

the chosen mode of delivery for this study due to the possibility to time delivery and placenta processing accordingly.

6.3.2.1. Inclusion criteria

I. Term pregnancy (≥37 week gestation)
II. Live birth delivery by elective Caesarean section
III. Maternal age 18-50 years

6.3.2.2. Exclusion criteria

I. Complicated pregnancy
II. Oligohydramnios
III. Pre-eclampsia
IV. Intra-uterine growth restriction (IUGR)
V. Abnormal placental perfusion – reduced, absent or reversed end diastolic flow on Doppler study
VI. Active smoker
VII. Maternal request to take placenta home

6.3.3. Recruitment process

Potential participants were approached by the research team. Previously, women undergoing elective Caesarean sections at the Royal London Hospital were invited to attend an antenatal clinic appointment 4 to 10 weeks before the operation date. Participants were approached and information sheets were disseminated at the clinic, followed by informed consent on the day of operation (information sheet and

consent form can be found in appendices 9 and 10 respectively). This antenatal clinic was discontinued during this study, so a different strategy was employed. With guidance from the Obstetrics team, the researcher approached and consented eligible participants on the morning of elective Caesarean sections.

6.3.4. Personal exposure to air pollution

6.3.4.1. Modelled exposure at home address

Using the LAQT as before, colleagues at King's College London estimated the mean PM and NO_2 concentrations based on participants' home postcodes, 12 months prior to the date when participants underwent elective Caesarean sections.

6.3.5. Placenta processing

Placentas were acquired immediately after the third stage of labour, following placental inspection by the attending midwife, to check for abnormalities. Placentas were transported on ice to the laboratory for immediate processing to ensure cell viability. Blood was aspirated from the umbilical cord for erythrocyte extraction using a density gradient medium, as described above (section 2.7.1). Placental tissue was separated from the membrane using a scalpel, and dissected into pieces of roughly 3 x 3 x 3 cm. Placental tissues were rinsed first in ultrapure ddH_2O (Barnstead™ NANOpure Diamond Life Sciences, California, USA), then in DPBS, followed by vigorously agitating in 6-well cell culture plates (Corning® Costar® , Corning, USA). Tissues were incubated in trypsin (Santa Cruz biotechnology, USA) and dispase (Sigma-Aldrich, Poole, UK) for 3 h at 37°C, 5% CO_2. Hyaluronidase (Sigma-Aldrich, Poole, UK) was added to the digestion mixture for the final 1 h. Digested placental

tissues passed through a 36 µm gauze (NITEX 03-36/28, Sefar, Switzerland), and were diluted with 1:1 DPBS and washed 3 times in DPBS. Finally, cells were resuspended in PBS/FBS (2%), according to consistency, for placental macrophage (PMac) extraction, using the human monocyte enrichment cocktail and cell-depleted erythrocytes as described previously (section 2.7.1.). Following enrichment, PMacs were resuspended in appropriate cell culture medium for investigation.

6.3.6. Presence of inhaled pollutants within the placenta

6.3.6.1. Placental macrophage black carbon analysis

As with alveolar macrophages, cytospin slides of PMacs were generated and analysed using the same image analytical method, under light microscopy. 1000 randomly selected PMacs from each sample were examined, and the mean BC content quantified using ImageJ, as described before (section 2.6.4.).

6.3.6.2. Electron microscopy of placental macrophages

Specimens for electron microscopy (EM) were fixed in phosphate buffered 4% glutaraldehyde (Agar Scientific, UK) then centrifuged at 2000 rpm (314 x g) for 10 min. The supernatant was discarded, the pellet was re-suspended in 1:1 2% low gel temperature agarose (VWR International, Pennsylvania, USA). Once the agarose had solidified, the block was cut into cubes of approximately 1 x 1 x 1 mm which were washed overnight in phosphate buffer. They were then post-fixed in 1% osmium tetroxide (Agar Scientific, Essex, UK), washed in phosphate buffer, dehydrated through graded ethanol, cleared in propylene oxide (Agar Scientific, Essex, UK) and embedded in Araldite epoxy resin (Agar Scientific, Essex, UK).

For light microscopy, semi-thin (0.5 mm) sections were mounted onto slides and stained with 1% toluidine blue in 1% borax (Atom Scientific, Cheshire, UK). For EM, ultrathin sections (60 – 90 nm) were mounted on grids and stained with saturated aqueous uranyl acetate (Agar Scientific, Essex, UK) followed by Reynold's lead citrate. The ultrathin sections were examined with a Jeol JEM1230 electron microscope and digital images captured on a SIS Morada camera. At least 20 individual PMacs were examined under EM per sample, images of cells with suspected intracellular carbonaceous material were captured for expert analysis. EM was conducted by a biomedical scientist at the Royal London Hospital.

6.3.6.2.1. Investigation of intravacuolar particles

EM images with suspicious black inclusions compatible with the appearance of carbonaceous PM were examined and analysed by Dr Carolyn Jones, an honorary research fellow and an experienced electron microscopist, specialising in the placenta, at St Mary's Hospital, Manchester, UK.

6.3.7. Placental macrophages function

To determine *in vitro* phagocytic ability of PMacs, enriched PMacs were exposed to diesel exhaust particle (SRM 2975, NIST, USA) as with alveolar macrophages.

6.3.7.1. Placental macrophage uptake of diesel exhaust particles

Nunc™Lab-Tek™ cell culture chambers (ThermoFisher Scientific, UK) were pre-coated in 1:10 collagen for at least 30 min at 37°C. The concentration used was lower than that for alveolar macrophage experiments due to the abundance of

PMacs available from each placenta. Enriched PMacs were washed and resuspended in complete RPMI before allowing to adhere in chamber wells overnight at 37^{o}C, 5% CO_2. PMacs were washed in incomplete RPMI the next day. Experimental chamber wells were exposed to 2 µg/mL of diesel exhaust particles (DEP) (SRM 2975, NIST, USA) for 2 h at 37°C, 5% CO_2; control wells were unexposed. Chamber wells were washed and stained as described before (section 2.7.2.). Light microscopy and image analysis of PMacs were performed using 20 randomly selected cells per culture (i.e. unexposed and exposed to DEP).

6.4. Results – Translocation of air pollution particles

6.4.1. Modelled exposure at home address

The participants all resided in east London and had similar exposure to air pollutants. Using the LAQT, the participants' (n=13) mean ± SEM annual PM_{10} and $PM_{2.5}$ exposure in the 12 months prior to delivery were 27.22 ± 0.60 μg/m^3 and 16.94 ± 0.38 μg/m^3 respectively (figure 6.1).

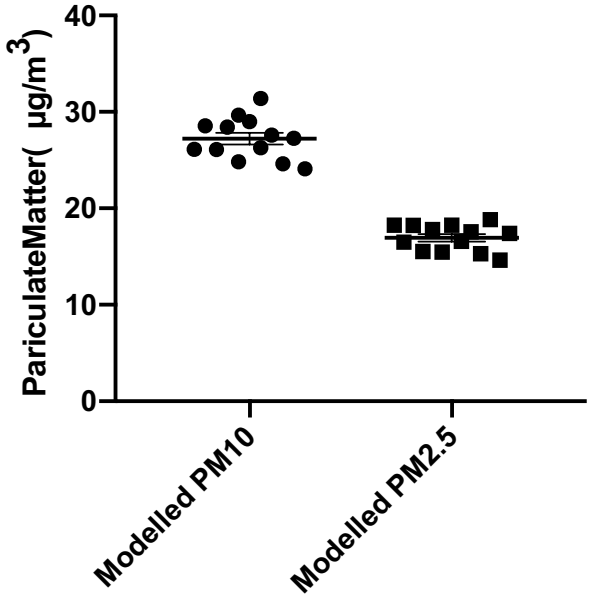

Figure 6.1. Modelled annual mean PM_{10} and $PM_{2.5}$ levels 12 months prior to delivery date, based on participants' home addresses. Bars represent standard error of mean.

Monitoring of personal air pollution exposure using aethalometers and nitrogen dioxide samplers was not performed for this part of the study – this is because participants were recruited on the day of delivery, rendering pre-delivery monitoring impossible; and their daily routines were greatly altered post-delivery. Nitrogen dioxide samplers were distributed to participants after delivery, with the instructions to carry them on their persons once normal daily routines had resumed, however, there was no return of any samplers – owing to loss or inconvenience.

6.5. Presence of inhaled pollutants within the placenta

6.5.1. Placental macrophage black carbon Analysis

Randomly selecting 1000 placental macrophages (PMacs) per placenta, a total of 15000 PMacs from 15 placentas were examined. All placentas contained suspicious black inclusions in at least 3 of the 1000 cells per placenta (figure 6.2, table 6.1). Mean ± SEM placental macrophage black carbon (PMacBC) was 0.02 ± 0.02 μm^2.

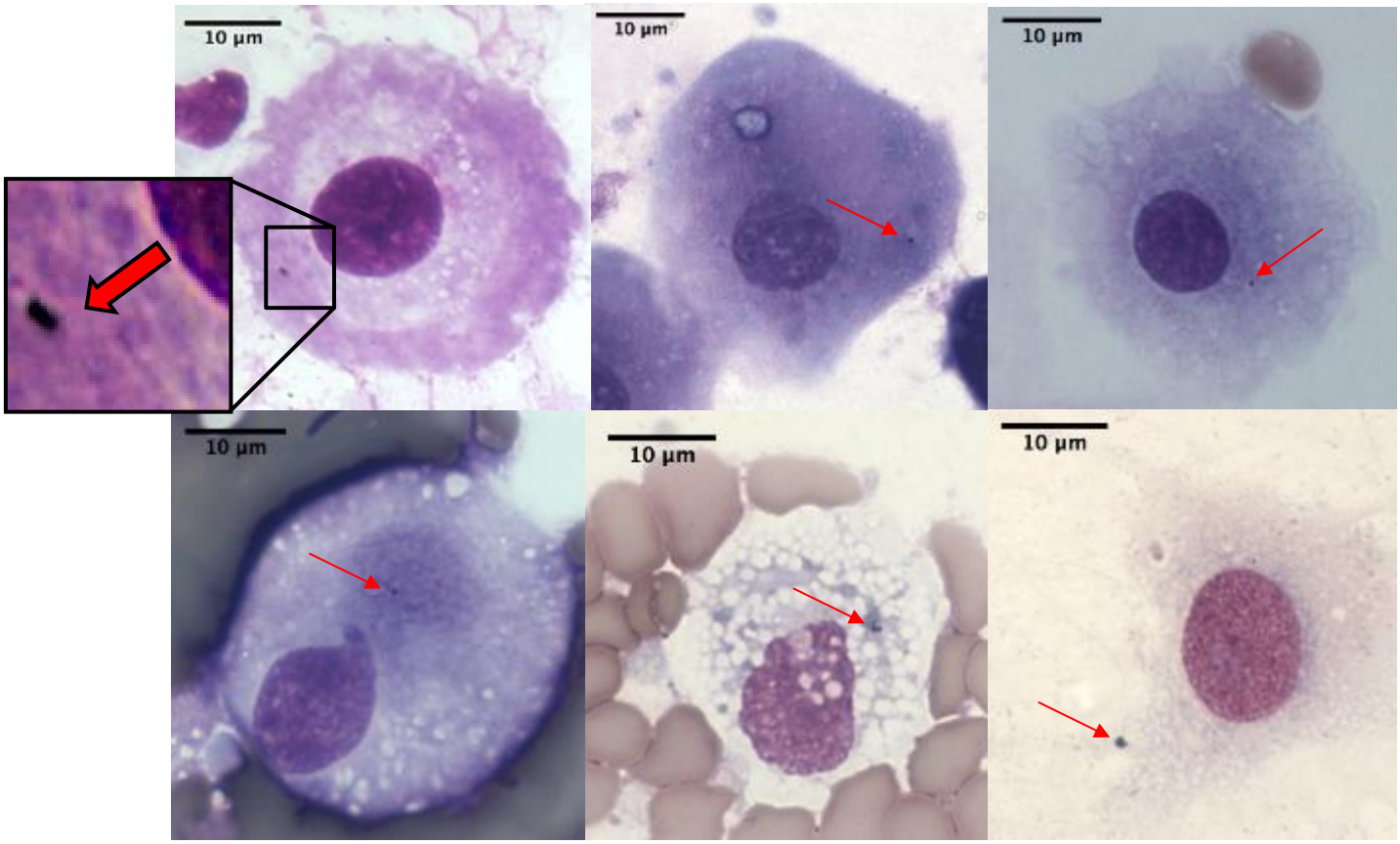

Figure 6.2. Selected light microscopy images of placental macrophages showing black inclusions (red arrows) compatible with the appearances of phagocytosed black carbon, as seen in alveolar macrophages in previous chapters.

Sample	Number of cells containing black inclusions	Number of inclusions in 1000 cells	Mean PMacBC (μm^2)	Modelled annual mean PM10 ($\mu g/m^3$)	Modelled annual mean PM2.5 ($\mu g/m^3$)
1	17	18	0.0044	28.43	18.81
2	10	11	0.0052	27.59	17.77
3	19	21	0.0129	28.56	18.23
4	7	7	0.0030	29.64	18.26
5	5	7	0.0014	28.97	18.25
6	9	15	0.0022	27.24	17.38
7	14	18	0.0028	26.07	16.59
8	13	13	0.0027	24.08	14.62
9	24	24	0.0068	26.13	15.53
10	4	5	0.0038	24.81	15.28
11	3	3	0.0015	N/A*	N/A*
12	8	11	0.0119	24.63	15.44
13	13	17	0.0026	31.37	17.56
14	2	2	0.0006	N/A*	N/A*
15	4	6	0.0017	26.29	16.49

Table 6 .1. Summary of number of PMac cells with black inclusions found in each placenta (1000 randomly selected cells examined per placenta), with the mean placental macrophage black carbon and the participants' modelled annual mean PM exposures.
* Two of the participants resided in newly developed areas where postcode modelling of PM exposure was not available.

No evidence of correlation was seen between modelled exposures to PM and PMacBC, with r=0.26, p=0.44 for PM_{10}; and r=0.24, *p*=0.48 for $PM_{2.5}$ (figure 6.3).

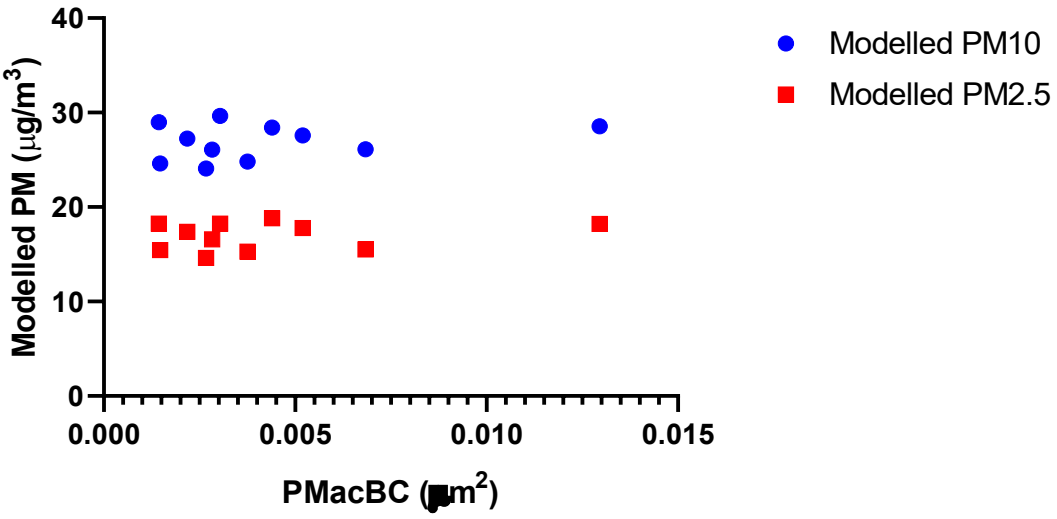

Figure 6.3. Spearman correlation between modelled PM (PM_{10} and $PM_{2.5}$) and placental macrophage black carbon (PMacBC): r=0.26, *p*=0.44 for PM_{10}; and r=0.24, p=0.48 for $PM_{2.5}$.

The poor correlation is likely to be related to the similar modelled PM exposure for all participants, given that they all resided in east London. The variation in PMacBC between participants is also small, as most PMacs reviewed contained no black carbon in the cytoplasm.

6.5.2. Electron microscopy of placental macrophages

Five placentas were further examined by electron microscopy, which demonstrated ultrafine and nano sized black inclusions (some measuring <0.1µm) within placental macrophages from each placenta (figure 6.4). These black inclusions had appearances compatible with diesel exhaust particles seen in PMacs exposed to DEP *in vitro* (figure 6.5), they also resemble phagocytosed black carbon seen in alveolar macrophages, as demonstrated in chapter 3 of this thesis.

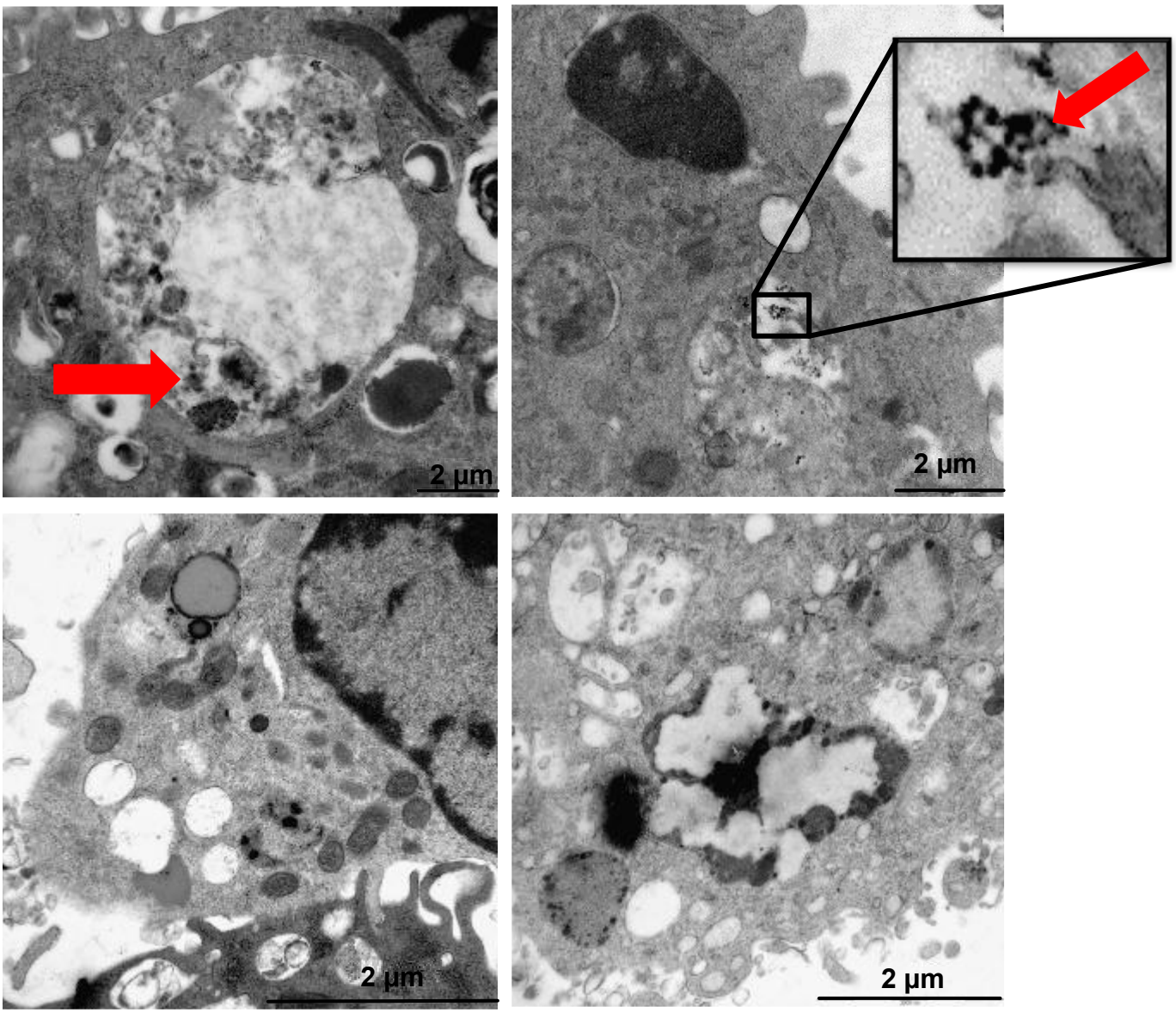

Figure 6.4. Electron microscopy images of placental macrophages showing black inclusions in vacuoles compatible with diesel exhaust particles appearances.

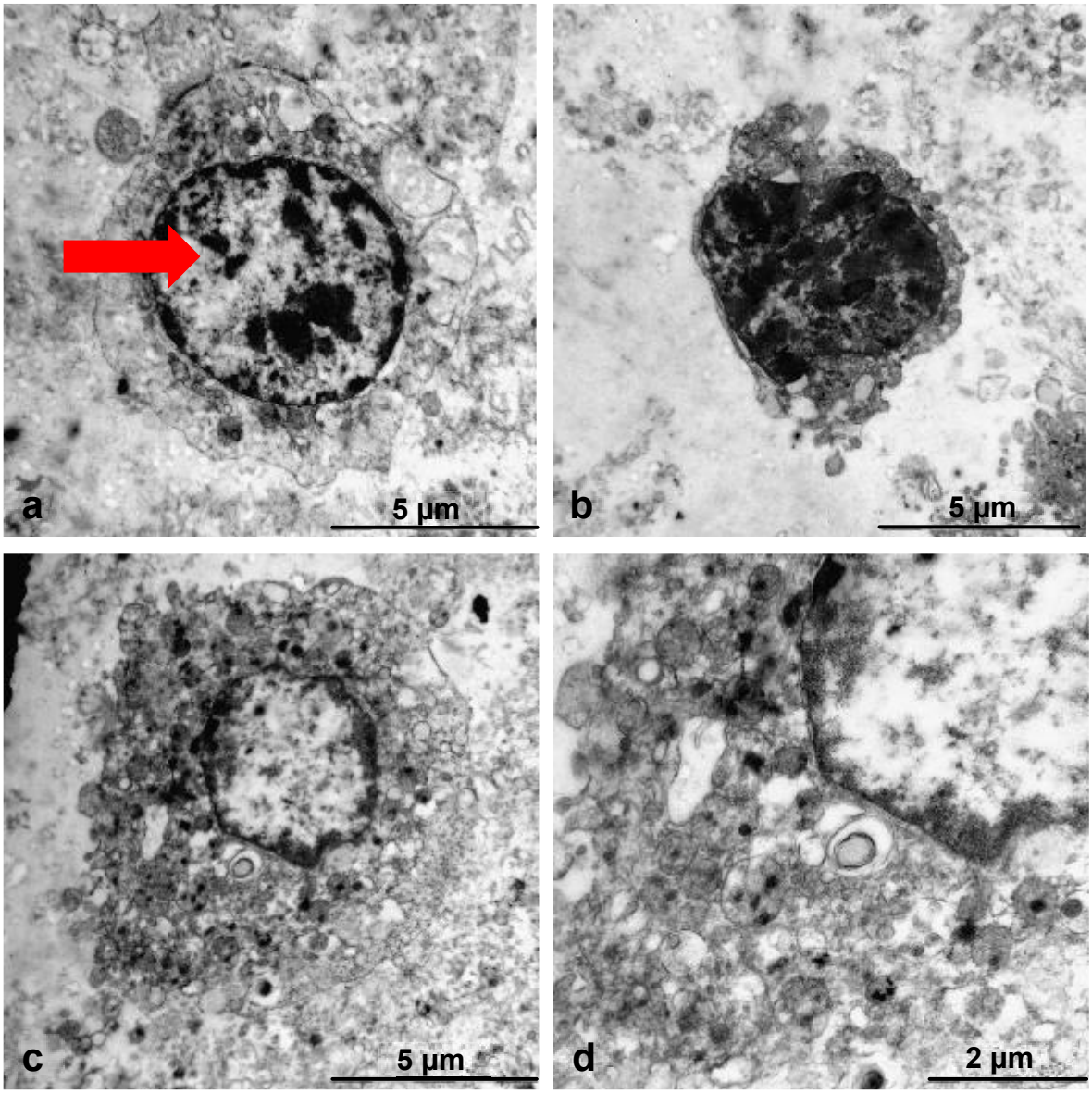

Figure 6.5. Electron microscopy images of placental macrophages exposed to diesel exhaust particles *in vitro*. (d) is an magnified image of (c).

6.6. Placental macrophage uptake of diesel exhaust particles *in vitro*

Placental macrophages demonstrated phagocytic ability *in vitro* when exposed to diesel exhaust particles (figure 6.6), median of differences (IQR) in PMacBC between unexposed and exposed PMacs was 5.96 (4.16 – 10.55) μm^2, n=13, p<0.001 (figure 6.7).

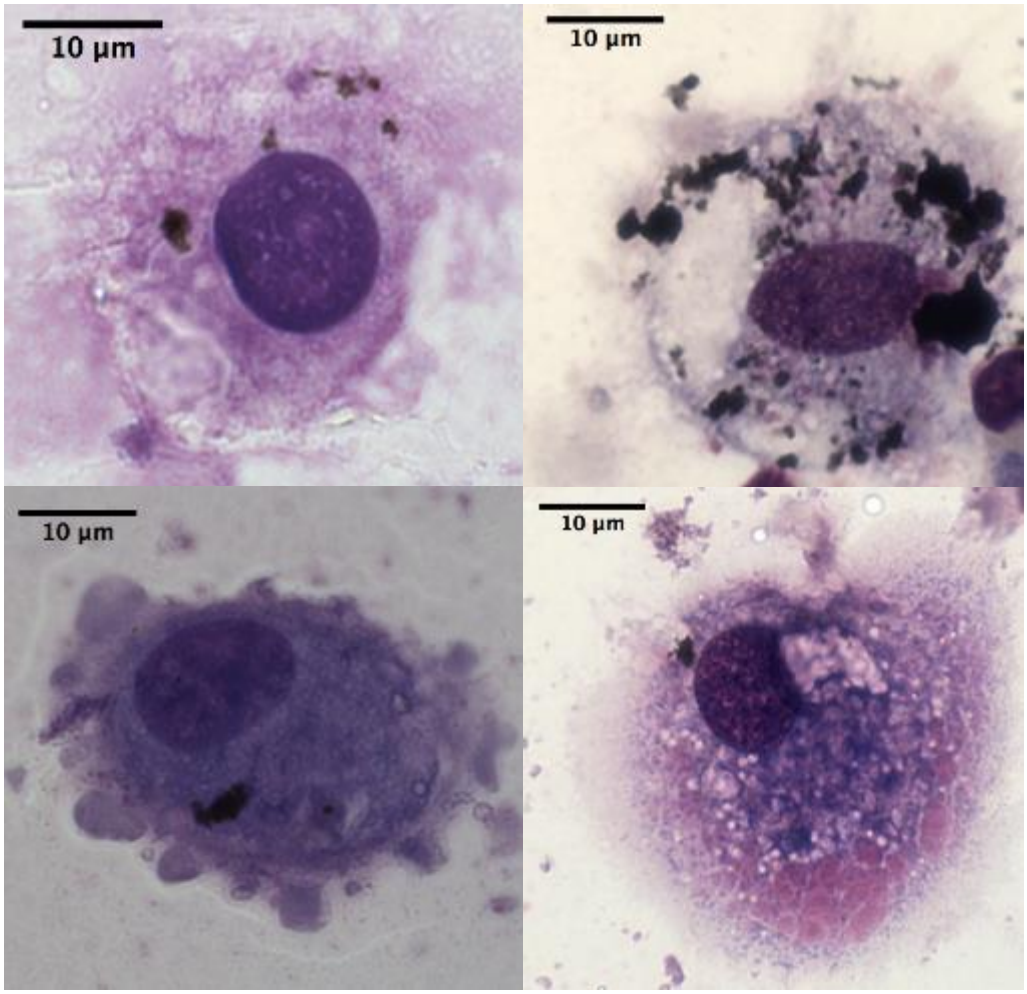

Figure 6.6. Light microscopy images of placental macrophages after exposure to diesel exhaust particles, demonstrating *in vitro* phagocytic ability.

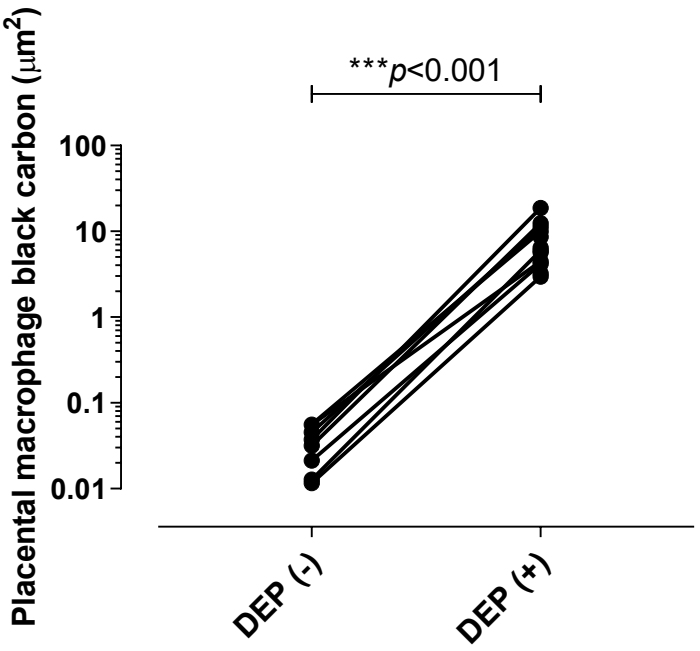

Figure 6.7. Placental macrophage black carbon in PMac cells unexposed (DEP-) and exposed (DEP+) to diesel exhaust particles, presented in log scale. Comparison by Wilcoxon test.

6.7. Discussion

Using light microscopy, evidence of black inclusions, with appearances compatible with inhaled black carbon seen in alveolar macrophages, were found in placental macrophages, suggesting that it is indeed possible for carbonaceous particles to translocate from the maternal lung via the circulation to the placenta. More importantly, this finding was universal, i.e. all placentas had evidence of such black inclusions. Since it is difficult to prove the nature of the black inclusions using only their appearances, electron microscopy was used to pursue any such black materials that were phagocytosed. Using light and electronic microscopy images of placental macrophages exposed to diesel exhaust particles *in vitro*, and resources available in the literature, suspicious black substances compatible with diesel exhaust particles were seen within placental macrophage vacuoles.

Combining both light and electron microscopy, these results strongly support that inhaled carbonaceous ultrafine PM can penetrate the tissue barrier of the respiratory system, invade the circulation and travel to distant organs. Notably, ultrafine particles were seen in placental macrophages – this is consistent with the findings from previous research that nano-sized particles are able to cross tissue barrier. As the amount of BC detected in the placenta is very small, whether it is adequate to cause significant problems in placental function and fetal development remains unknown. However, particles able to reach the placenta may also cross over the placental barrier and reach the fetus – an area which warrants future investigations.

6.7.1. Strengths

For the first time, using visualisation by microscopy, this work demonstrated the possibility of inhaled ultrafine carbonaceous particles to escape the airway immune system, penetrate tissue barriers, and reach the systemic circulation, traveling to a distant destination in the body. This also provides a potential mechanism of how air pollution can affect the unborn fetus, resulting in adverse birth outcomes. Two different methods, light and electron microscopy, were used to confirm the nature of the black inclusions identified.

6.7.2. Limitations

The number of participants was small and they were exposed to similar amount of air pollutants as they were all residing in similar parts of London. The study would be strengthened by a larger number of participants, ideally from different parts of the country, or different countries, with different levels of air pollution. The study would also be improved by comparing PMacBC to maternal AMBC. Active smoking, but not passive smoking, was an exclusion criterion. Both active and passive smoking are known to have negative effects on maternal and fetal health, it would therefore be reasonable to consider the effects of passive smoking on placental macrophage function and carbon loading.

6.7.3. Summary

Using light and electron microscopy, the results from this chapter show evidence of carbonaceous ultrafine particles in placental macrophages in all placentas examined. These findings suggest it is possible for ultrafine particles to translocate

from the lung to distant organs, providing a potential mechanism of how air pollution can affect extra-pulmonary systems, and how the unborn fetus is impacted.

Chapter 7: Discussion

7. Discussion

7.1. Key findings

In this thesis, I sought to address the mechanisms underlying the vulnerability of children with cystic fibrosis to air pollution. Air pollution is harmful to all individuals at every stage of the life course – with children, particularly those with respiratory conditions, being most susceptible. Phagocytosis by alveolar macrophages, a major route of clearing inhaled carbonaceous particulate matter, is impaired in CF. This was demonstrated by the reduced amount of phagocytosed black carbon observed in AMs from children with CF, despite children from both CF and control groups were exposed to similar levels of PM and NO_2, as seen in their modelled and personal monitoring data. However, the impairment of CF AM function appeared to be only an *in vivo* problem, as AM phagocytosis of DEP in both CF and control groups were similar *in vitro*. This reinforced the hypothesis that it is the CF airway environment that hinders AM phagocytosis. Brugha *et al.* [102] suggested that impaired AM phagocytosis in severe asthma was likely to be secondary to increased production of PGE_2, PGE_2 was therefore speculated to be the driver of AM phagocytosis impairment in CF as well.

The work in this thesis showed that not only did the CF group have higher levels of urinary metabolites of PGD_2, PGE_2 and PGJ_2, reflecting increased production of these PGs; PGE_2 level in sputum supernatant was also higher in the CF group compared to healthy controls. Additionally, the increased expression of COX-2 in CF AMs further supplemented the evidence for increased PGs production.

The *in vitro* assay showed that PGE_2 did inhibit responder AM phagocytosis, which was reversed by blocking PGE_2 with an EP2 receptor antagonist.

Notably, CF sputum supernatant also exhibited inhibitory effects on responder AM phagocytosis *in vitro*; and reversal of this inhibition was achieved using an EP2 receptor antagonist, strongly suggesting PGE_2 as a driving factor for the functional impairment in CF AMs.

This thesis then explored the consequences of impaired phagocytosis of inhaled PM. The protective role of functional AM was demonstrated – the absence or dysfunction of AMs (e.g. when responder AMs were inhibited by PGE_2) allowed PM to invade and affect epithelial cells. While the epithelium-macrophage *in vitro* model may not be a true representation of the airway, it functioned as a proof-of-concept model nonetheless. The invasion of residual PM in the airway into epithelial cells may trigger local inflammation – as seen in the increased IL-8 release from epithelial cells when exposed to DEP.

This thesis went on to investigate further consequences of non-phagocytosed PM. PM may remain in the airway for various reasons, such as impaired AM phagocytosis, or saturation of AM functional capacity in heavily polluted environment. Ultrafine and nano-particles have been shown to be able to penetrate tissue barriers in animal and human *ex vivo* models. The work in this thesis showed, for the first time, microscopy evidence of particle translocation from the lung to distant organ. The placenta was not only used as a proxy for distant organs,

presence of pollutant particles in the placenta may also explain the association between maternal air pollution exposure and adverse birth outcomes.

All participating women were exposed to similar levels of pollution based on their modelled exposure data, and suspicious black inclusions were identified in all placentas. The black inclusions have appearances compatible with inhaled carbonaceous particles phagocytosed by AMs, as shown by the sputum work on AMBC in this thesis, and also in the literature [102,103]. Electron micrographs of placental macrophages revealed black substances, which were compatible with carbonaceous ultrafine particles in AMs demonstrated by Bunn *et al.* [194]. To further support these findings, PMacs were exposed to DEP *in vitro* – this demonstrated the phagocytic ability of PMacs, and at the same time, electron microscopy of PMacs exposed to DEP showed that the characters of DEP do akin to the black substances observed within unexposed PMac.

Figure 7.1 demonstrates the overall findings of this thesis.

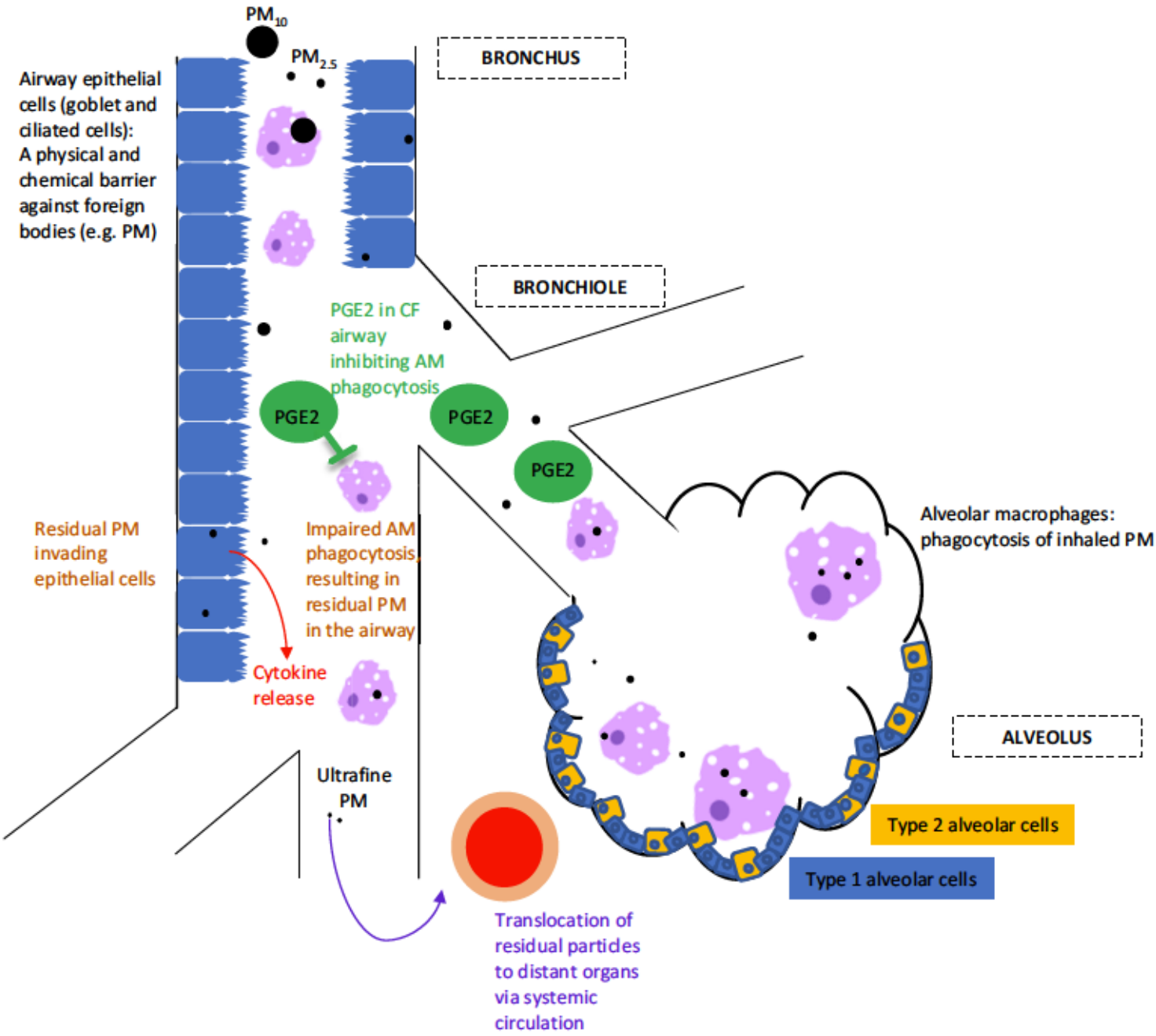

Figure 7.1. Overall findings of this thesis. Inhaled carbonaceous particulate matter (PM) reaching the lower airway, which is protected by the physical and chemical barrier of airway epithelial cells; inhaled particles (PM_{10} and $PM_{2.5}$) are phagocytosed by functional alveolar macrophages (AM), which are migrating upwards along the bronchial tree. Prostaglandin E2 (PGE_2), produced by AMs and other nucleated cells, reduces the functional capacity of alveolar macrophages, thereby leaving inhaled PM non-phagocytosed. Residual PM in the airway then invade epithelial cells, causing release of cytokines (e.g. IL-8). Ultrafine non-phagocytosed PM can then translocate to distant organs via the systemic circulation, exerting their toxicity directly at the distant site.

7.2. Implication for future studies

Since it was impossible to compare the amount of AMs present in the CF vs healthy airways, future studies examining AMBC using bronchoalveolar lavage might be of interest. Lavage can be performed under a more controlled environment using a fixed amount of saline flush. The difficulty with this approach is that it is uncommon for healthy children to undergo bronchoscopy.

To better model the CF airways, primary epithelial cells can be used. Further studies that explore the complex interactions of cytokines released from all airway cells under the influence of diesel exhaust particles are needed.

The work in this thesis shows that a short 3-day course of Ibuprofen can reduce PG metabolites in the urine, reflecting a reduction in PG production. However, a longer term, randomised study spanning beyond the average half-life of AM will be beneficial and more compelling, where AMBC can be compared before and after intervention with Ibuprofen. While air pollution remains a global public health issue, and air quality improvement is slow to progress, the current generation of children continue to suffer the health effects of air pollution. Cyclooxygenase inhibitors such as Ibuprofen may offer a therapeutic option in tackling inflammation caused by air pollution.

Intracellular clusters of bacteria were observed within AMs from children with CF. These children were clinically stable at the time and had no evidence of concurrent infections or exacerbations. While it is known in the literature that some bacteria are able to bypass the immune system and survive within macrophages, it may be

important to identify any concealed bacteria residing within cells, and understand the consequence of such uninvited residence – for example, whether these bacteria are driving chronic but subclinical airway inflammation. During chest exacerbations, patients with CF can be asymptomatic but have declined lung function. Sputum and cough swabs cultures often show no microbiology growth, and therefore antibiotic treatments are commonly empirical. Any pathogens concealed within alveolar macrophages might not be detected by routine hospital cultures. Using the monocyte enrichment method, AMs can be isolated and intracellular contents may be cultured. This will not only allow for more tailored antibiotic treatments, it may also identify patients who are asymptomatic but at risk of colonisation. Further studies in this area are definitely warranted.

With the knowledge that the handling of inhaled carbonaceous particles is compromised in patients with CF, subsequently worsening the pre-existing chronic airway inflammation, it is important to raise patients' awareness and advise them accordingly, so they can take appropriate measures to reduce their personal exposure to air pollution. While air quality activists worldwide are striving for cleaner air, which is an urgent matter as no child should be denied their fundamental right to live in a clean environment, the responsibility largely lies with policy makers and governmental bodies, and the process of reducing air pollutants to target levels could be protracted. It is therefore sensible to pursue therapeutic avenues which may protect vulnerable children from the harmful effects of air pollutants. With the techniques used in this work, it is possible to non-invasively isolate primary alveolar macrophages from children, allowing further studies on how to improve their phagocytic function. To limit, halt, or reverse any lung damage caused by air

pollution, that is occurring on a day to day basis, will allow our current generation of children to achieve their developmental potential, lead a healthy childhood, and improve the quality of life of those with existing respiratory illnesses.

References

8. References

1. WHO. *WHO Europe: Health Effects of Particulate Matter*. 2013.
2. WHO Global Urban Ambient Air Pollution Database. http://www.who.int/phe/health_topics/outdoorair/databases/cities/en/.
3. Holegate S, Grigg J, Raymond A, et al. Every Breath We Take: The Lifelong Impact of Air Pollution. 2016.
4. Newby DE, Mannucci PM, Tell GS, et al. Expert position paper on air pollution and cardiovascular disease. *European heart journal.* 2015;36(2):83-93b.
5. Review of evidence on health aspects of air pollution – REVIHAAP Project. . 2013; http://www.euro.who.int/__data/assets/pdf_file/0004/193108/REVIHAAP-Final-technical-report-final-version.pdf?ua=1.
6. Guarnieri M, Balmes JR. Outdoor air pollution and asthma. *Lancet.* 2014;383(9928):1581-1592.
7. EU emission inventory report 1990-2016. 2016.
8. Sources of air pollutants. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, No 109.* 2016.
9. Schauer J.J. RWF, Hildemann, L.M., Mazurek M.A., Cass G.R., Simoneit B.R.R. Source apportionment of airborne particulate matter using organic compounds as tracers. *Atmospheric Environment.* 1996;30(22):3837-3855.
10. Nunes RAO, Alvim-Ferraz MCM, Martins FG, Sousa SIV. Assessment of shipping emissions on four ports of Portugal. *Environmental pollution.* 2017;231(Pt 2):1370-1379.
11. WHO. Air pollution and child health. Prescribing clean air. 2018.
12. Air Quality Expert Group: Nitrogen Dioxide in the United Kingdom. 2004.
13. *New Car CO2 Report 2017.* 2017.
14. Internation Agency for Research on Cancer WHO. IARC: Diesel engine exhaust carcinogenic. 2012.
15. Organization WH. Air Quality Guidelines for Europe. *WHO Regional Publications, European Series.* 2000;No. 91.
16. Haq IJ, Gray MA, Garnett JP, Ward C, Brodlie M. Airway surface liquid homeostasis in cystic fibrosis: pathophysiology and therapeutic targets. *Thorax.* 2016;71(3):284-287.
17. Tarran R. Regulation of airway surface liquid volume and mucus transport by active ion transport. *Proc Am Thorac Soc.* 2004;1(1):42-46.
18. Nicod LP. Lung defences: an overview. *European respiratory review : an official journal of the European Respiratory Society.* 2005;14(95):45-50.
19. Mills PR, Davies RJ, Devalia JL. Airway epithelial cells, cytokines, and pollutants. *American journal of respiratory and critical care medicine.* 1999;160(5 Pt 2):S38-43.
20. Bayram H, Devalia JL, Sapsford RJ, et al. The effect of diesel exhaust particles on cell function and release of inflammatory mediators from human bronchial epithelial cells in vitro. *American journal of respiratory cell and molecular biology.* 1998;18(3):441-448.
21. Devalia JL, Sapsford RJ, Cundell DR, Rusznak C, Campbell AM, Davies RJ. Human bronchial epithelial cell dysfunction following in vitro exposure to nitrogen dioxide. *The European respiratory journal.* 1993;6(9):1308-1316.
22. Brugha R, Edmondson C, Davies JC. Outdoor air pollution and cystic fibrosis. *Paediatric respiratory reviews.* 2018;28:80-86.
23. Provoost S, Maes T, Willart MA, Joos GF, Lambrecht BN, Tournoy KG. Diesel exhaust particles stimulate adaptive immunity by acting on pulmonary dendritic cells. *Journal of immunology.* 2010;184(1):426-432.
24. Lee J, Breton G, Oliveira TY, et al. Restricted dendritic cell and monocyte progenitors in human cord blood and bone marrow. *The Journal of experimental medicine.* 2015;212(3):385-399.

25. Yang J, Zhang L, Yu C, Yang XF, Wang H. Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. *Biomark Res.* 2014;2(1):1.
26. Boyette LB, Macedo C, Hadi K, et al. Phenotype, function, and differentiation potential of human monocyte subsets. *PloS one.* 2017;12(4):e0176460.
27. Draijer C, Peters-Golden M. Alveolar Macrophages in Allergic Asthma: the Forgotten Cell Awakes. *Current allergy and asthma reports.* 2017;17(2):12.
28. !!! INVALID CITATION !!! {}.
29. Kaur M, Bell T, Salek-Ardakani S, Hussell T. Macrophage adaptation in airway inflammatory resolution. *European respiratory review : an official journal of the European Respiratory Society.* 2015;24(137):510-515.
30. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep.* 2014;6:13.
31. Tarique AA, Sly PD, Holt PG, et al. CFTR-dependent defect in alternatively-activated macrophages in cystic fibrosis. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society.* 2017;16(4):475-482.
32. Maus UA, Janzen S, Wall G, et al. Resident alveolar macrophages are replaced by recruited monocytes in response to endotoxin-induced lung inflammation. *Am J Respir Cell Mol Biol.* 2006;35(2):227-235.
33. Roszer T. Understanding the Biology of Self-Renewing Macrophages. *Cells.* 2018;7(8).
34. Male D, Brostoff, J., Roth, D., Roitt, I. Mononuclear phagocytes in immune defense: Immunology 8th Edition.
35. Cooper GM. The Cell: A Molecular Approach. 2nd edition. Sunderland (MA): Sinauer Associates; 2000. Endocytosis. Available from: https://www.ncbi.nlm.nih.gov/books/NBK9831/.
36. Crotzer VL, Blum JS. Autophagy and its role in MHC-mediated antigen presentation. *Journal of immunology.* 2009;182(6):3335-3341.
37. Arango Duque G, Descoteaux A. Macrophage cytokines: involvement in immunity and infectious diseases. *Frontiers in immunology.* 2014;5:491.
38. Park SY, Byun EJ, Lee JD, Kim S, Kim HS. Air Pollution, Autophagy, and Skin Aging: Impact of Particulate Matter (PM10) on Human Dermal Fibroblasts. *International journal of molecular sciences.* 2018;19(9).
39. Hsu YH, Chuang HC, Lee YH, et al. Traffic-related particulate matter exposure induces nephrotoxicity in vitro and in vivo. *Free radical biology & medicine.* 2019;135:235-244.
40. Hilbi H, Zychlinsky A, Sansonetti PJ. Macrophage apoptosis in microbial infections. *Parasitology.* 1997;115 Suppl:S79-87.
41. Boylen CE, Sly PD, Zosky GR, Larcombe AN. Physiological and inflammatory responses in an anthropomorphically relevant model of acute diesel exhaust particle exposure are sex and dose-dependent. *Inhalation toxicology.* 2011;23(14):906-917.
42. Saxena RK, Gilmour MI, Hays MD. Isolation and quantitative estimation of diesel exhaust and carbon black particles ingested by lung epithelial cells and alveolar macrophages in vitro. *BioTechniques.* 2008;44(6):799-805.
43. Korten I, Ramsey K, Latzin P. Air pollution during pregnancy and lung development in the child. *Paediatric respiratory reviews.* 2017;21:38-46.
44. Morales E, Garcia-Esteban R, de la Cruz OA, et al. Intrauterine and early postnatal exposure to outdoor air pollution and lung function at preschool age. *Thorax.* 2015;70(1):64-73.
45. Proietti E, Roosli M, Frey U, Latzin P. Air pollution during pregnancy and neonatal outcome: a review. *Journal of aerosol medicine and pulmonary drug delivery.* 2013;26(1):9-23.
46. Pedersen M, Giorgis-Allemand L, Bernard C, et al. Ambient air pollution and low birthweight: a European cohort study (ESCAPE). *The Lancet Respiratory medicine.* 2013;1(9):695-704.
47. Commission E. Air Quality Standards.

48. Familari M, Nääv Å, Erlandsson L, et al. Exposure of trophoblast cells to fine particulate matter air pollution leads to growth inhibition, inflammation and ER stress. *PLoS One.* 2019;14(7):e0218799.
49. Kim W, Cho Y, Song MK, et al. Effect of particulate matter 2.5 on gene expression profile and cell signaling in JEG-3 human placenta cells. *Environmental toxicology.* 2018;33(11):1123-1134.
50. Maghbooli Z, Hossein-Nezhad A, Adabi E, et al. Air pollution during pregnancy and placental adaptation in the levels of global DNA methylation. *PloS one.* 2018;13(7):e0199772.
51. Jung CR, Chen WT, Tang YH, Hwang BF. Fine particulate matter exposure during pregnancy and infancy and incident asthma. *The Journal of allergy and clinical immunology.* 2019;143(6):2254-2262 e2255.
52. Latzin P, Roosli M, Huss A, Kuehni CE, Frey U. Air pollution during pregnancy and lung function in newborns: a birth cohort study. *The European respiratory journal.* 2009;33(3):594-603.
53. Carrillo G, Perez Patron MJ, Johnson N, Zhong Y, Lucio R, Xu X. Asthma prevalence and school-related hazardous air pollutants in the US-Mexico border area. *Environmental research.* 2017;162:41-48.
54. Lee JY, Leem JH, Kim HC, et al. Effects of traffic-related air pollution on susceptibility to infantile bronchiolitis and childhood asthma: A cohort study in Korea. *The Journal of asthma : official journal of the Association for the Care of Asthma.* 2017:1-8.
55. Girguis MS, Strickland MJ, Hu X, et al. Exposure to acute air pollution and risk of bronchiolitis and otitis media for preterm and term infants. *Journal of exposure science & environmental epidemiology.* 2017.
56. Chen Z, Salam MT, Eckel SP, Breton CV, Gilliland FD. Chronic effects of air pollution on respiratory health in Southern California children: findings from the Southern California Children's Health Study. *Journal of thoracic disease.* 2015;7(1):46-58.
57. Hwang BF, Chen YH, Lin YT, Wu XT, Leo Lee Y. Relationship between exposure to fine particulates and ozone and reduced lung function in children. *Environmental research.* 2015;137:382-390.
58. Gasana J, Dillikar D, Mendy A, Forno E, Ramos Vieira E. Motor vehicle air pollution and asthma in children: a meta-analysis. *Environmental research.* 2012;117:36-45.
59. Gauderman WJ, Vora H, McConnell R, et al. Effect of exposure to traffic on lung development from 10 to 18 years of age: a cohort study. *Lancet.* 2007;369(9561):571-577.
60. Urman R, McConnell R, Islam T, et al. Associations of children's lung function with ambient air pollution: joint effects of regional and near-roadway pollutants. *Thorax.* 2014;69(6):540-547.
61. Gauderman WJ, Urman R, Avol E, et al. Association of improved air quality with lung development in children. *The New England journal of medicine.* 2015;372(10):905-913.
62. Poynter ME, Persinger RL, Irvin CG, et al. Nitrogen dioxide enhances allergic airway inflammation and hyperresponsiveness in the mouse. *American journal of physiology Lung cellular and molecular physiology.* 2006;290(1):L144-152.
63. Bouazza N, Foissac F, Urien S, et al. Fine particulate pollution and asthma exacerbations. *Archives of disease in childhood.* 2017.
64. Darrow LA, Klein M, Flanders WD, Mulholland JA, Tolbert PE, Strickland MJ. Air pollution and acute respiratory infections among children 0-4 years of age: an 18-year time-series study. *American journal of epidemiology.* 2014;180(10):968-977.
65. Garcia E, Berhane KT, Islam T, et al. Association of Changes in Air Quality With Incident Asthma in Children in California, 1993-2014. *Jama.* 2019;321(19):1906-1915.
66. Pope CA, 3rd. Respiratory hospital admissions associated with PM10 pollution in Utah, Salt Lake, and Cache Valleys. *Archives of environmental health.* 1991;46(2):90-97.
67. Saenen ND, Plusquin M, Bijnens E, et al. In Utero Fine Particle Air Pollution and Placental Expression of Genes in the Brain-Derived Neurotrophic Factor Signaling Pathway: An

ENVIRONAGE Birth Cohort Study. *Environmental health perspectives.* 2015;123(8):834-840.
68. Bos I, De Boever P, Int Panis L, Meeusen R. Physical activity, air pollution and the brain. *Sports medicine.* 2014;44(11):1505-1518.
69. Basagaña X, Esnaola M, Rivas I, et al. Neurodevelopmental Deceleration by Urban Fine Particles from Different Emission Sources: A Longitudinal Observational Study. *Environ Health Perspect.* 2016;124(10):1630-1636.
70. Pujol J, Martinez-Vilavella G, Macia D, et al. Traffic pollution exposure is associated with altered brain connectivity in school children. *NeuroImage.* 2016;129:175-184.
71. Sram RJ, Veleminsky M, Jr., Veleminsky M, Sr., Stejskalova J. The impact of air pollution to central nervous system in children and adults. *Neuro endocrinology letters.* 2017;38(6):389-396.
72. Forns J, Dadvand P, Esnaola M, et al. Longitudinal association between air pollution exposure at school and cognitive development in school children over a period of 3.5 years. *Environmental research.* 2017;159:416-421.
73. Risnes KR, Vatten LJ, Baker JL, et al. Birthweight and mortality in adulthood: a systematic review and meta-analysis. *International journal of epidemiology.* 2011;40(3):647-661.
74. Calderon-Garciduenas L, Vincent R, Mora-Tiscareno A, et al. Elevated plasma endothelin-1 and pulmonary arterial pressure in children exposed to air pollution. *Environmental health perspectives.* 2007;115(8):1248-1253.
75. Bilenko N, van Rossem L, Brunekreef B, et al. Traffic-related air pollution and noise and children's blood pressure: results from the PIAMA birth cohort study. *European journal of preventive cardiology.* 2015;22(1):4-12.
76. Park SK. Ambient Air Pollution and Type 2 Diabetes: Do the Metabolic Effects of Air Pollution Start Early in Life? *Diabetes.* 2017;66(7):1755-1757.
77. Thiering E, Cyrys J, Kratzsch J, et al. Long-term exposure to traffic-related air pollution and insulin resistance in children: results from the GINIplus and LISAplus birth cohorts. *Diabetologia.* 2013;56(8):1696-1704.
78. Lakey PS, Berkemeier T, Tong H, et al. Chemical exposure-response relationship between air pollutants and reactive oxygen species in the human respiratory tract. *Scientific reports.* 2016;6:32916.
79. Lodovici M, Bigagli E. Oxidative stress and air pollution exposure. *Journal of toxicology.* 2011;2011:487074.
80. Gurgueira SA, Lawrence J, Coull B, Murthy GG, Gonzalez-Flecha B. Rapid increases in the steady-state concentration of reactive oxygen species in the lungs and heart after particulate air pollution inhalation. *Environmental health perspectives.* 2002;110(8):749-755.
81. Siponen T, Yli-Tuomi T, Aurela M, et al. Source-specific fine particulate air pollution and systemic inflammation in ischaemic heart disease patients. *Occupational and environmental medicine.* 2015;72(4):277-283.
82. Ruckerl R, Greven S, Ljungman P, et al. Air pollution and inflammation (interleukin-6, C-reactive protein, fibrinogen) in myocardial infarction survivors. *Environmental health perspectives.* 2007;115(7):1072-1080.
83. Tsai DH, Riediker M, Berchet A, et al. Effects of short- and long-term exposures to particulate matter on inflammatory marker levels in the general population. *Environmental science and pollution research international.* 2019.
84. Watanabe M, Noma H, Kurai J, et al. Effects of Short-Term Exposure to Particulate Air Pollutants on the Inflammatory Response and Respiratory Symptoms: A Panel Study in Schoolchildren from Rural Areas of Japan. *International journal of environmental research and public health.* 2016;13(10).
85. Farina F, Lonati E, Milani C, et al. In Vivo Comparative Study on Acute and Sub-acute Biological Effects Induced by Ultrafine Particles of Different Anthropogenic Sources in BALB/c Mice. *International journal of molecular sciences.* 2019;20(11).
86. Kim JA, Cho JH, Park IH, Shin JM, Lee SA, Lee HM. Diesel Exhaust Particles Upregulate Interleukins IL-6 and IL-8 in Nasal Fibroblasts. *PloS one.* 2016;11(6):e0157058.

87. Stearns RC, Paulauskis JD, Godleski JJ. Endocytosis of ultrafine particles by A549 cells. *American journal of respiratory cell and molecular biology.* 2001;24(2):108-115.
88. Thorley AJ, Ruenraroengsak P, Potter TE, Tetley TD. Critical determinants of uptake and translocation of nanoparticles by the human pulmonary alveolar epithelium. *ACS nano.* 2014;8(11):11778-11789.
89. Oliveira VR, Uriarte JJ, Falcones B, et al. Biomechanical Response of Lung Epithelial Cells to Iron Oxide and Titanium Dioxide Nanoparticles. *Frontiers in physiology.* 2019;10:1047.
90. Nemmar A, Beegam S, Yuvaraju P, Yasin J, Shahin A, Ali BH. Interaction of amorphous silica nanoparticles with erythrocytes in vitro: role of oxidative stress. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology.* 2014;34(2):255-265.
91. Oberdorster G, Sharp Z, Atudorei V, et al. Extrapulmonary translocation of ultrafine carbon particles following whole-body inhalation exposure of rats. *Journal of toxicology and environmental health Part A.* 2002;65(20):1531-1543.
92. Campagnolo L, Massimiani M, Vecchione L, et al. Silver nanoparticles inhaled during pregnancy reach and affect the placenta and the foetus. *Nanotoxicology.* 2017;11(5):687-698.
93. Aengenheister L, Dietrich D, Sadeghpour A, et al. Gold nanoparticle distribution in advanced in vitro and ex vivo human placental barrier models. *J Nanobiotechnology.* 2018;16(1):79.
94. Muoth C, Grossgarten M, Karst U, et al. Impact of particle size and surface modification on gold nanoparticle penetration into human placental microtissues. *Nanomedicine.* 2017;12(10):1119-1133.
95. Wick P, Malek A, Manser P, et al. Barrier capacity of human placenta for nanosized materials. *Environmental health perspectives.* 2010;118(3):432-436.
96. Elder A, Gelein R, Silva V, et al. Translocation of inhaled ultrafine manganese oxide particles to the central nervous system. *Environmental health perspectives.* 2006;114(8):1172-1178.
97. Maher BA, Ahmed IA, Karloukovski V, et al. Magnetite pollution nanoparticles in the human brain. *Proceedings of the National Academy of Sciences of the United States of America.* 2016;113(39):10797-10801.
98. Greater London Authority. Guide for Monitoring Air Quality in London. . 2018.
99. Brauer M. How much, how long, what, and where: air pollution exposure assessment for epidemiologic studies of respiratory disease. *Proc Am Thorac Soc.* 2010;7(2):111-115.
100. Alexis NE, Lay JC, Zeman KL, Geiser M, Kapp N, Bennett WD. In vivo particle uptake by airway macrophages in healthy volunteers. *American journal of respiratory cell and molecular biology.* 2006;34(3):305-313.
101. Kulkarni N, Pierse N, Rushton L, Grigg J. Carbon in airway macrophages and lung function in children. *The New England journal of medicine.* 2006;355(1):21-30.
102. Brugha RE, Mushtaq N, Round T, et al. Carbon in airway macrophages from children with asthma. *Thorax.* 2014;69(7):654-659.
103. Bai Y, Brugha RE, Jacobs L, Grigg J, Nawrot TS, Nemery B. Carbon loading in airway macrophages as a biomarker for individual exposure to particulate matter air pollution - A critical review. *Environment international.* 2015;74:32-41.
104. Bai Y, Bove H, Nawrot TS, Nemery B. Carbon load in airway macrophages as a biomarker of exposure to particulate air pollution; a longitudinal study of an international Panel. *Particle and fibre toxicology.* 2018;15(1):14.
105. Tillery A. Black Carbon Loading in Sputum and Bronchoalveolar Lavage Macrophages as a Biomarker of Air Pollution Exposure. 2019.
106. Saenen ND, Bove H, Steuwe C, et al. Children's Urinary Environmental Carbon Load. A Novel Marker Reflecting Residential Ambient Air Pollution Exposure? *American journal of respiratory and critical care medicine.* 2017;196(7):873-881.
107. Laybourn-Langton L, Quilter-Pinner, H., Ho, H. Lethal and Illegal: Solving London's Air Pollution Crisis. 2016.

108. Scotet V, Dugueperoux I, Saliou P, et al. Evidence for decline in the incidence of cystic fibrosis: a 35-year observational study in Brittany, France. *Orphanet journal of rare diseases.* 2012;7:14.
109. Ratjen F, Doring G. Cystic fibrosis. *Lancet.* 2003;361(9358):681-689.
110. Vankeerberghen A, Cuppens H, Cassiman JJ. The cystic fibrosis transmembrane conductance regulator: an intriguing protein with pleiotropic functions. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society.* 2002;1(1):13-29.
111. Mall MA, Hartl D. CFTR: cystic fibrosis and beyond. *The European respiratory journal.* 2014;44(4):1042-1054.
112. Sly PD, Gangell CL, Chen L, et al. Risk factors for bronchiectasis in children with cystic fibrosis. *N Engl J Med.* 2013;368(21):1963-1970.
113. Cystic Fibrosis Mutation Database.
114. Estrada-Veras J, Groninger H. Palliative care for patients with cystic fibrosis #265. *Journal of palliative medicine.* 2013;16(4):446-447.
115. Boucher RC. Airway surface dehydration in cystic fibrosis: pathogenesis and therapy. *Annu Rev Med.* 2007;58:157-170.
116. Cantin AM, Hartl D, Konstan MW, Chmiel JF. Inflammation in cystic fibrosis lung disease: Pathogenesis and therapy. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society.* 2015;14(4):419-430.
117. De Rose V. Mechanisms and markers of airway inflammation in cystic fibrosis. *The European respiratory journal.* 2002;19(2):333-340.
118. Pezzulo AA, Tang XX, Hoegger MJ, et al. Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung. *Nature.* 2012;487(7405):109-113.
119. Boucher RC. New concepts of the pathogenesis of cystic fibrosis lung disease. *The European respiratory journal.* 2004;23(1):146-158.
120. Bruscia EM, Bonfield TL. Cystic Fibrosis Lung Immunity: The Role of the Macrophage. *J Innate Immun.* 2016;8(6):550-563.
121. Di A, Brown ME, Deriy LV, et al. CFTR regulates phagosome acidification in macrophages and alters bactericidal activity. *Nature cell biology.* 2006;8(9):933-944.
122. Alexis NE, Muhlebach MS, Peden DB, Noah TL. Attenuation of host defense function of lung phagocytes in young cystic fibrosis patients. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society.* 2006;5(1):17-25.
123. Wright AK, Rao S, Range S, et al. Pivotal Advance: Expansion of small sputum macrophages in CF: failure to express MARCO and mannose receptors. *Journal of leukocyte biology.* 2009;86(3):479-489.
124. Hodge S, Upham JW, Pizzutto S, et al. Is Alveolar Macrophage Phagocytic Dysfunction in Children With Protracted Bacterial Bronchitis a Forerunner to Bronchiectasis? *Chest.* 2016;149(2):508-515.
125. Painter RG, Marrero L, Lombard GA, Valentine VG, Nauseef WM, Wang G. CFTR-mediated halide transport in phagosomes of human neutrophils. *J Leukoc Biol.* 2010;87(5):933-942.
126. Ratner D, Mueller C. Immune responses in cystic fibrosis: are they intrinsically defective? *American journal of respiratory cell and molecular biology.* 2012;46(6):715-722.
127. Rajan S, Saiman L. Pulmonary infections in patients with cystic fibrosis. *Semin Respir Infect.* 2002;17(1):47-56.
128. Malhotra S, Hayes D, Jr., Wozniak DJ. Cystic Fibrosis and Pseudomonas aeruginosa: the Host-Microbe Interface. *Clin Microbiol Rev.* 2019;32(3).
129. Stutts MJ, Knowles MR, Gatzy JT, Boucher RC. Oxygen consumption and ouabain binding sites in cystic fibrosis nasal epithelium. *Pediatric research.* 1986;20(12):1316-1320.
130. Lau GW, Hassett DJ, Ran H, Kong F. The role of pyocyanin in Pseudomonas aeruginosa infection. *Trends in molecular medicine.* 2004;10(12):599-606.
131. Valvano MA. Intracellular survival of Burkholderia cepacia complex in phagocytic cells. *Canadian journal of microbiology.* 2015;61(9):607-615.

132. Mitchell G, Chen C, Portnoy DA. Strategies Used by Bacteria to Grow in Macrophages. *Microbiology spectrum.* 2016;4(3).
133. Martin DW, Mohr CD. Invasion and intracellular survival of Burkholderia cepacia. *Infection and immunity.* 2000;68(1):24-29.
134. Schwab U, Abdullah LH, Perlmutt OS, et al. Localization of Burkholderia cepacia complex bacteria in cystic fibrosis lungs and interactions with Pseudomonas aeruginosa in hypoxic mucus. *Infection and immunity.* 2014;82(11):4729-4745.
135. Elliott TR, Rayment NB, Hudspith BN, et al. Lamina propria macrophage phenotypes in relation to Escherichia coli in Crohn's disease. *BMC gastroenterology.* 2015;15:75.
136. Verhaeghe C, Delbecque K, de Leval L, Oury C, Bours V. Early inflammation in the airways of a cystic fibrosis foetus. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society.* 2007;6(4):304-308.
137. Chen J, Jiang XH, Chen H, et al. CFTR negatively regulates cyclooxygenase-2-PGE(2) positive feedback loop in inflammation. *Journal of cellular physiology.* 2012;227(6):2759-2766.
138. Elizur A, Cannon CL, Ferkol TW. Airway inflammation in cystic fibrosis. *Chest.* 2008;133(2):489-495.
139. Meyer KC, Lewandoski JR, Zimmerman JJ, Nunley D, Calhoun WJ, Dopico GA. Human neutrophil elastase and elastase/alpha 1-antiprotease complex in cystic fibrosis. Comparison with interstitial lung disease and evaluation of the effect of intravenously administered antibiotic therapy. *The American review of respiratory disease.* 1991;144(3 Pt 1):580-585.
140. Roca-Ferrer J, Pujols L, Gartner S, et al. Upregulation of COX-1 and COX-2 in nasal polyps in cystic fibrosis. *Thorax.* 2006;61(7):592-596.
141. Aronoff DM, Canetti C, Peters-Golden M. Prostaglandin E2 inhibits alveolar macrophage phagocytosis through an E-prostanoid 2 receptor-mediated increase in intracellular cyclic AMP. *Journal of immunology.* 2004;173(1):559-565.
142. Czerska K, Sobczynska-Tomaszewska A, Sands D, et al. Prostaglandin-endoperoxide synthase genes COX1 and COX2 - novel modifiers of disease severity in cystic fibrosis patients. *Journal of applied genetics.* 2010;51(3):323-330.
143. Jabr S, Gartner S, Milne GL, et al. Quantification of major urinary metabolites of PGE2 and PGD2 in cystic fibrosis: correlation with disease severity. *Prostaglandins, leukotrienes, and essential fatty acids.* 2013;89(2-3):121-126.
144. Konstan MW, Walenga RW, Hilliard KA, Hilliard JB. Leukotriene B4 markedly elevated in the epithelial lining fluid of patients with cystic fibrosis. *The American review of respiratory disease.* 1993;148(4 Pt 1):896-901.
145. Lucidi V, Ciabattoni G, Bella S, Barnes PJ, Montuschi P. Exhaled 8-isoprostane and prostaglandin E(2) in patients with stable and unstable cystic fibrosis. *Free radical biology & medicine.* 2008;45(6):913-919.
146. Zakrzewski JT, Barnes NC, Piper PJ, Costello JF. Detection of sputum eicosanoids in cystic fibrosis and in normal saliva by bioassay and radioimmunoassay. *British journal of clinical pharmacology.* 1987;23(1):19-27.
147. Yang J, Eiserich JP, Cross CE, Morrissey BM, Hammock BD. Metabolomic profiling of regulatory lipid mediators in sputum from adult cystic fibrosis patients. *Free radical biology & medicine.* 2012;53(1):160-171.
148. Strandvik B, Svensson E, Seyberth HW. Prostanoid biosynthesis in patients with cystic fibrosis. *Prostaglandins, leukotrienes, and essential fatty acids.* 1996;55(6):419-425.
149. Medeiros A, Peres-Buzalaf C, Fortino Verdan F, Serezani CH. Prostaglandin E2 and the suppression of phagocyte innate immune responses in different organs. *Mediators Inflamm.* 2012;2012:327568.
150. Zaslona Z, Serezani CH, Okunishi K, Aronoff DM, Peters-Golden M. Prostaglandin E2 restrains macrophage maturation via E prostanoid receptor 2/protein kinase A signaling. *Blood.* 2012;119(10):2358-2367.

151. Dehle FC, Mukaro VR, Jurisevic C, et al. Defective lung macrophage function in lung cancer +/- chronic obstructive pulmonary disease (COPD/emphysema)-mediated by cancer cell production of PGE2? *PloS one.* 2013;8(4):e61573.
152. Rossi AG, McCutcheon JC, Roy N, Chilvers ER, Haslett C, Dransfield I. Regulation of macrophage phagocytosis of apoptotic cells by cAMP. *Journal of immunology.* 1998;160(7):3562-3568.
153. Pereira PAT, Assis PA, Prado MKB, et al. Prostaglandins D2 and E2 have opposite effects on alveolar macrophages infected with Histoplasma capsulatum. *Journal of lipid research.* 2018;59(2):195-206.
154. Pereira PA, Bitencourt Cda S, dos Santos DF, Nicolete R, Gelfuso GM, Faccioli LH. Prostaglandin D2-loaded microspheres effectively activate macrophage effector functions. *European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences.* 2015;78:132-139.
155. Simeone AM, Nieves-Alicea R, McMurtry VC, Colella S, Krahe R, Tari AM. Cyclooxygenase-2 uses the protein kinase C/ interleukin-8/urokinase-type plasminogen activator pathway to increase the invasiveness of breast cancer cells. *Int J Oncol.* 2007;30(4):785-792.
156. Rosen BH, Evans TIA, Moll SR, et al. Infection is Not Required for Mucoinflammatory Lung Disease in CFTR-knockout Ferrets. *American journal of respiratory and critical care medicine.* 2018.
157. Weiss G, Schaible UE. Macrophage defense mechanisms against intracellular bacteria. *Immunological reviews.* 2015;264(1):182-203.
158. Dean TP, Dai Y, Shute JK, Church MK, Warner JO. Interleukin-8 concentrations are elevated in bronchoalveolar lavage, sputum, and sera of children with cystic fibrosis. *Pediatric research.* 1993;34(2):159-161.
159. Lynch JP, 3rd, Sayah DM, Belperio JA, Weigt SS. Lung transplantation for cystic fibrosis: results, indications, complications, and controversies. *Seminars in respiratory and critical care medicine.* 2015;36(2):299-320.
160. Konstan MW, Byard PJ, Hoppel CL, Davis PB. Effect of high-dose ibuprofen in patients with cystic fibrosis. *N Engl J Med.* 1995;332(13):848-854.
161. Lands LC, Milner R, Cantin AM, Manson D, Corey M. High-dose ibuprofen in cystic fibrosis: Canadian safety and effectiveness trial. *J Pediatr.* 2007;151(3):249-254.
162. Konstan MW, VanDevanter DR, Sawicki GS, et al. Association of High-Dose Ibuprofen Use, Lung Function Decline, and Long-Term Survival in Children with Cystic Fibrosis. *Annals of the American Thoracic Society.* 2018.
163. Goeminne PC, Kicinski M, Vermeulen F, et al. Impact of air pollution on cystic fibrosis pulmonary exacerbations: a case-crossover analysis. *Chest.* 2013;143(4):946-954.
164. Psoter KJ, De Roos AJ, Mayer JD, Kaufman JD, Wakefield J, Rosenfeld M. Fine particulate matter exposure and initial Pseudomonas aeruginosa acquisition in cystic fibrosis. *Annals of the American Thoracic Society.* 2015;12(3):385-391.
165. Psoter KJ, De Roos AJ, Wakefield J, Mayer JD, Rosenfeld M. Air pollution exposure is associated with MRSA acquisition in young U.S. children with cystic fibrosis. *BMC pulmonary medicine.* 2017;17(1):106.
166. Goss CH, Newsom SA, Schildcrout JS, Sheppard L, Kaufman JD. Effect of ambient air pollution on pulmonary exacerbations and lung function in cystic fibrosis. *American journal of respiratory and critical care medicine.* 2004;169(7):816-821.
167. Geiser M, Stoeger T, Casaulta M, et al. Biokinetics of nanoparticles and susceptibility to particulate exposure in a murine model of cystic fibrosis. *Particle and fibre toxicology.* 2014;11:19.
168. Fujii T, Hayashi S, Hogg JC, Vincent R, Van Eeden SF. Particulate matter induces cytokine expression in human bronchial epithelial cells. *American journal of respiratory cell and molecular biology.* 2001;25(3):265-271.
169. Kunzi L, Krapf M, Daher N, et al. Toxicity of aged gasoline exhaust particles to normal and diseased airway epithelia. *Scientific reports.* 2015;5:11801.

170. Jeannet N, Fierz M, Schneider S, et al. Acute toxicity of silver and carbon nanoaerosols to normal and cystic fibrosis human bronchial epithelial cells. *Nanotoxicology.* 2016;10(3):279-291.
171. Qu F, Qin XQ, Cui YR, et al. Ozone stress down-regulates the expression of cystic fibrosis transmembrane conductance regulator in human bronchial epithelial cells. *Chemico-biological interactions.* 2009;179(2-3):219-226.
172. Miller MR, Hankinson J, Brusasco V, et al. Standardisation of spirometry. *The European respiratory journal.* 2005;26(2):319-338.
173. Gibson PG, Grootendor DC, Henry RL, et al. Sputum induction in children. *The European respiratory journal Supplement.* 2002;37:44s-46s.
174. Brightling CE, Ward R, Woltmann G, et al. Induced sputum inflammatory mediator concentrations in eosinophilic bronchitis and asthma. *American journal of respiratory and critical care medicine.* 2000;162(3 Pt 1):878-882.
175. Lee J, Banu SK, Burghardt RC, Starzinski-Powitz A, Arosh JA. Selective inhibition of prostaglandin E2 receptors EP2 and EP4 inhibits adhesion of human endometriotic epithelial and stromal cells through suppression of integrin-mediated mechanisms. *Biol Reprod.* 2013;88(3):77.
176. Duan M, Steinfort DP, Smallwood D, et al. CD11b immunophenotyping identifies inflammatory profiles in the mouse and human lungs. *Mucosal Immunol.* 2016;9(2):550-563.
177. Grosser T, Naji A, FitzGerald GA. Urinary Prostaglandin Metabolites: An Incomplete Reckoning and a Flush to Judgment. *Circulation research.* 2018;122(4):537-539.
178. Efthimiadis A, Spanevello A, Hamid Q, et al. Methods of sputum processing for cell counts, immunocytochemistry and in situ hybridisation. *The European respiratory journal Supplement.* 2002;37:19s-23s.
179. Hubeau C, Puchelle E, Gaillard D. Distinct pattern of immune cell population in the lung of human fetuses with cystic fibrosis. *J Allergy Clin Immunol.* 2001;108(4):524-529.
180. Brennan S, Sly PD, Gangell CL, et al. Alveolar macrophages and CC chemokines are increased in children with cystic fibrosis. *Eur Respir J.* 2009;34(3):655-661.
181. Floto RA, Olivier KN, Saiman L, et al. US Cystic Fibrosis Foundation and European Cystic Fibrosis Society consensus recommendations for the management of non-tuberculous mycobacteria in individuals with cystic fibrosis: executive summary. *Thorax.* 2016;71(1):88-90.
182. Ryu YJ, Koh WJ, Daley CL. Diagnosis and Treatment of Nontuberculous Mycobacterial Lung Disease: Clinicians' Perspectives. *Tuberc Respir Dis (Seoul).* 2016;79(2):74-84.
183. Domingo-Gonzalez R, Katz S, Serezani CH, Moore TA, Levine AM, Moore BB. Prostaglandin E2-induced changes in alveolar macrophage scavenger receptor profiles differentially alter phagocytosis of Pseudomonas aeruginosa and Staphylococcus aureus post-bone marrow transplant. *Journal of immunology.* 2013;190(11):5809-5817.
184. Lands LC, Dauletbaev N. High-Dose Ibuprofen in Cystic Fibrosis. *Pharmaceuticals (Basel).* 2010;3(7):2213-2224.
185. Lands LC, Stanojevic S. Oral non-steroidal anti-inflammatory drug therapy for lung disease in cystic fibrosis. *Cochrane Database Syst Rev.* 2016;4:CD001505.
186. Rezaee F, Georas SN. Breaking barriers. New insights into airway epithelial barrier function in health and disease. *Am J Respir Cell Mol Biol.* 2014;50(5):857-869.
187. Doornaert B, Leblond V, Galiacy S, et al. Negative impact of DEP exposure on human airway epithelial cell adhesion, stiffness, and repair. *Am J Physiol Lung Cell Mol Physiol.* 2003;284(1):L119-132.
188. Cohen-Cymberknoh M, Kerem E, Ferkol T, Elizur A. Airway inflammation in cystic fibrosis: molecular mechanisms and clinical implications. *Thorax.* 2013;68(12):1157-1162.
189. Jundi K, Greene CM. Transcription of Interleukin-8: How Altered Regulation Can Affect Cystic Fibrosis Lung Disease. *Biomolecules.* 2015;5(3):1386-1398.
190. Sagel SD, Kapsner R, Osberg I, Sontag MK, Accurso FJ. Airway inflammation in children with cystic fibrosis and healthy children assessed by sputum induction. *American journal of respiratory and critical care medicine.* 2001;164(8 Pt 1):1425-1431.

191. Mayer-Hamblett N, Aitken ML, Accurso FJ, et al. Association between pulmonary function and sputum biomarkers in cystic fibrosis. *Am J Respir Crit Care Med.* 2007;175(8):822-828.
192. Massengale AR, Quinn F, Yankaskas J, et al. Reduced interleukin-8 production by cystic fibrosis airway epithelial cells. *Am J Respir Cell Mol Biol.* 1999;20(5):1073-1080.
193. DiMango E, Zar HJ, Bryan R, Prince A. Diverse Pseudomonas aeruginosa gene products stimulate respiratory epithelial cells to produce interleukin-8. *The Journal of clinical investigation.* 1995;96(5):2204-2210.
194. Bunn HJ, Dinsdale D, Smith T, Grigg J. Ultrafine particles in alveolar macrophages from normal children. *Thorax.* 2001;56(12):932-934.
195. Gauthier TW, Grunwell JR, Ping XD, Harris FL, Brown LA. Impaired defenses of neonatal mouse alveolar macrophage with cftr deletion are modulated by glutathione and TGFbeta1. *Physiological reports.* 2017;5(6).
196. Entezari M, Weiss DJ, Sitapara R, et al. Inhibition of high-mobility group box 1 protein (HMGB1) enhances bacterial clearance and protects against Pseudomonas Aeruginosa pneumonia in cystic fibrosis. *Molecular medicine.* 2012;18:477-485.
197. Vandivier RW, Fadok VA, Ogden CA, et al. Impaired clearance of apoptotic cells from cystic fibrosis airways. *Chest.* 2002;121(3 Suppl):89S.
198. Vandivier RW, Fadok VA, Hoffmann PR, et al. Elastase-mediated phosphatidylserine receptor cleavage impairs apoptotic cell clearance in cystic fibrosis and bronchiectasis. *The Journal of clinical investigation.* 2002;109(5):661-670.
199. Fick RB, Jr., Naegel GP, Matthay RA, Reynolds HY. Cystic fibrosis pseudomonas opsonins. Inhibitory nature in an in vitro phagocytic assay. *The Journal of clinical investigation.* 1981;68(4):899-914.
200. Cassino RJ, Sordelli DO, Macri CN, Kohan M, Dillon MH, Pivetta OH. Pulmonary nonspecific defense mechanisms in cystic fibrosis. I. Phagocytic capacity of alveolar macrophages and neutrophils. *Pediatric research.* 1980;14(11):1212-1215.
201. Thomassen MJ, Demko CA, Wood RE, et al. Ultrastructure and function of alveolar macrophages from cystic fibrosis patients. *Pediatric research.* 1980;14(5):715-721.
202. Salina AC, Souza TP, Serezani CH, Medeiros AI. Efferocytosis-induced prostaglandin E2 production impairs alveolar macrophage effector functions during Streptococcus pneumoniae infection. *Innate immunity.* 2017;23(3):219-227.
203. Hubbard LL, Wilke CA, White ES, Moore BB. PTEN limits alveolar macrophage function against Pseudomonas aeruginosa after bone marrow transplantation. *American journal of respiratory cell and molecular biology.* 2011;45(5):1050-1058.
204. Medeiros AI, Serezani CH, Lee SP, Peters-Golden M. Efferocytosis impairs pulmonary macrophage and lung antibacterial function via PGE2/EP2 signaling. *The Journal of experimental medicine.* 2009;206(1):61-68.
205. Canetti C, Serezani CH, Atrasz RG, White ES, Aronoff DM, Peters-Golden M. Activation of phosphatase and tensin homolog on chromosome 10 mediates the inhibition of FcgammaR phagocytosis by prostaglandin E2 in alveolar macrophages. *Journal of immunology.* 2007;179(12):8350-8356.
206. Ballinger MN, Aronoff DM, McMillan TR, et al. Critical role of prostaglandin E2 overproduction in impaired pulmonary host response following bone marrow transplantation. *Journal of immunology.* 2006;177(8):5499-5508.
207. Canning BJ, Hmieleski RR, Spannhake EW, Jakab GJ. Ozone reduces murine alveolar and peritoneal macrophage phagocytosis: the role of prostanoids. *The American journal of physiology.* 1991;261(4 Pt 1):L277-282.
208. Laegreid WW, Liggitt HD, Silflow RM, Evermann JR, Taylor SM, Leid RW. Reversal of virus-induced alveolar macrophage bactericidal dysfunction by cyclooxygenase inhibition in vitro. *Journal of leukocyte biology.* 1989;45(4):293-300.
209. Kopp BT, Thompson R, Kim J, et al. Secondhand smoke alters arachidonic acid metabolism and inflammation in infants and children with cystic fibrosis. *Thorax.* 2019;74(3):237-246.

210. Ni I, Ji C, Vij N. Second-hand cigarette smoke impairs bacterial phagocytosis in macrophages by modulating CFTR dependent lipid-rafts. *PloS one.* 2015;10(3):e0121200.
211. Farhat SCL, Almeida MB, Silva-Filho L, Farhat J, Rodrigues JC, Braga ALF. Ozone is associated with an increased risk of respiratory exacerbations in patients with cystic fibrosis. *Chest.* 2013;144(4):1186-1192.
212. Jassal MS, Yu AM, Bhatia R, Keens TG, Davidson Ward SL. Effect of residential proximity to major roadways on cystic fibrosis exacerbations. *International journal of environmental health research.* 2013;23(2):119-131.
213. Kamdar O, Le W, Zhang J, Ghio AJ, Rosen GD, Upadhyay D. Air pollution induces enhanced mitochondrial oxidative stress in cystic fibrosis airway epithelium. *FEBS letters.* 2008;582(25-26):3601-3606.
214. Hurley MN, McKeever TM, Prayle AP, Fogarty AW, Smyth AR. Rate of improvement of CF life expectancy exceeds that of general population--observational death registration study. *J Cyst Fibros.* 2014;13(4):410-415.
215. Rao S, Grigg J. New insights into pulmonary inflammation in cystic fibrosis. *Archives of disease in childhood.* 2006;91(9):786-788.
216. Nwokoro C, Ewin C, Harrison C, et al. Cycling to work in London and inhaled dose of black carbon. *The European respiratory journal.* 2012;40(5):1091-1097.
217. Simpson JL, Gibson PG, Yang IA, et al. Impaired macrophage phagocytosis in non-eosinophilic asthma. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* 2013;43(1):29-35.
218. Arredouani M, Yang Z, Ning Y, et al. The scavenger receptor MARCO is required for lung defense against pneumococcal pneumonia and inhaled particles. *The Journal of experimental medicine.* 2004;200(2):267-272.
219. Hempel SL, Monick MM, Hunninghake GW. Lipopolysaccharide induces prostaglandin H synthase-2 protein and mRNA in human alveolar macrophages and blood monocytes. *The Journal of clinical investigation.* 1994;93(1):391-396.
220. Suri R, Periselneris J, Lanone S, et al. Exposure to welding fumes and lower airway infection with Streptococcus pneumoniae. *The Journal of allergy and clinical immunology.* 2015.
221. Mushtaq N, Ezzati M, Hall L, et al. Adhesion of Streptococcus pneumoniae to human airway epithelial cells exposed to urban particulate matter. *The Journal of allergy and clinical immunology.* 2011;127(5):1236-1242 e1232.
222. Klein SG, Serchi T, Hoffmann L, Blomeke B, Gutleb AC. An improved 3D tetraculture system mimicking the cellular organisation at the alveolar barrier to study the potential toxic effects of particles on the lung. *Particle and fibre toxicology.* 2013;10:31.
223. Mastalerz L, Celejewska-Wojcik N, Wojcik K, et al. Induced sputum supernatant bioactive lipid mediators can identify subtypes of asthma. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology.* 2015;45(12):1779-1789.

Appendix 1 – Summary of literature review on alveolar macrophage function in cystic fibrosis

Study	Subjects (source of AM)	Findings
Gauthier *et al.*, 2017 [195]	CFTR knock-out neonatal mice	CFTR knock-out AM showed reduced phagocytosis of *Staphylococcus aureus.*
Entezari et al., 2012 [196]	Human (BAL), and CFTR knock-out mice	High-mobility group box 1 protein (HMGB1) levels were elevated in CF; which impaired macrophage phagocytosis of *Pseudomonas aeruginosa*.
Wright *et al.*, 2009 [123]	Human (IS)	Expansion of small macrophages, with reduced expression of MARCO and CD206 in CF sputum macrophages, resulting in reduced phagocytosis of unopsonised fluorescent particles.
Di *et al.*, 2006 [121]	Human (BAL), and CFTR knock-out mice	CFTR deficient AMs showed a loss of subcellular acidification, resulting in enhanced survival of *Pseudomonas aeruginosa* within the phagosomal compartment.
Vandivier *et al.*, 2002 [197]	Human (IS), human monocyte-derived macrophages (HMDMs)	Increased number of *in vivo* apoptotic cells in CF sputum, suggesting defective airway clearance. CF sol inhibited HMDM phagocytosis of apoptotic cells *in vitro*, secondary to protease cleavage of cell surface receptor and deficiency in surfactant proteins.
Vandivier *et al.*, 2002 [198]	Human (BAL)	CF airway fluids inhibited AM removal of apoptotic cells *in vitro*, mediated by elastase facilitated phagocyte receptor cleavage.
Fick *et al.*, 1983 [199]	Human (BAL)	Impaired macrophage intracellular bacterial killing from CF-derived opsonins, associated with disrupted attachment of antibodies to the AM membranes.
Cassino *et al.*, 1980 [200]	Human (BAL)	Phagocytic capacity of AMs when exposed to *Candida albicans* was higher in CF.
Thomassen et al., 1980 [201]	Human (BAL)	Phagocytosis of *Pseudomonas aeruginosa* in normal serum was seen in both CF and normal AM; but phagocytosis was impaired when AMs were exposed to CF serum, except for phagocytosis of *Staphylococcus*.

Appendix 2 – Summary of literature review on the effects of prostaglandin E2 on alveolar macrophage function

Study	Subjects (source of AM)	Findings
Pereira *et al.*, 2018 [153]	Rats	PGE_2 decreased phagocytosis and killing of serum-opsonized *Histoplasma capsulatum,* with opposite effects seen with PGD_2.
Salina *et al.*, 2017 [202]	Rats	PGE_2 produced by AMs impaired clearance of non-opsonized *Streptococcus pneumoniae*.
Domingo-Gonzalez *et al.*, 2013 [183]	Bone marrow transplant (BMT) mice	PGE_2-driven alterations in scavenger receptor and miR-155 expression could inhibit bacterial killing, affecting phagocytosis of *Pseudomonas aeruginosa* and *Staphylococcus aureus.*
Hubbard *et al.*, 2011 [203]	Bone marrow transplant (BMT) mice	Post BMT overproduction of PGE_2 inhibited opsonised and unopsonised phagocytosis of *Pseudomonas aeruginosa*.
Medeiros *et al.*, 2009 [204]	Mice and rats	Apoptotic cells suppressed *in vitro* AM phagocytosis and bacterial killing. Intrapulmonary administration of apoptotic cells led to PGE_2 generation, resulting in impaired recruitment of leukocytes and clearance of *Streptococcus pneumoniae*
Canetti *et al.*, 2007[205]	Rats	PGE_2, by increasing the activity of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) in AMs, inhibited AM immune signalling. PTEN inhibition decreased PGE_2-induced suppression of AM killing of *Klebsiella pneumoniae*.
Ballinger *et al.*, 2006 [206]	Bone marrow transplant (BMT) mice	Increased production of PGE_2 post-BMT impaired host defence against *Escherichia coli.*
Canning *et al*., 1991 [207]	Mice	PGE_2 levels were increased following ozone exposure, associated with reduced phagocytosis of opsonized sheep red blood cells. Pre-treatment with COX-inhibitor (indomethacin) partially inhibited ozone-induced suppression of AM phagocytosis.
Laegreid *et al.*, 1989 [208]	Cattle	AMs were infected parainfluenza-3 (PI3 virus) virus, followed by exposure to *Staphylococcus epidermidis*. PI3 virus-infected AM had impaired bacterial killing, which was reversed by COX- inhibitor. *In vitro* impaired bactericidal function associated with virus infection of AM was associated with increased prostaglandins.

Appendix 3 – Summary of literature review on the effects of air pollution on cystic fibrosis

Study	Study design	Findings
Kopp *et al.*, 2019 [209]	Cohort study of 77 children with CF aged <10 years.	Children with CF who are exposed to second-hand smoke had altered arachidonic acid metabolism and inflammatory gene expression, resulting in impaired bacterial clearance.
Psoter *et al.*, 2017 [165]	Retrospective study in 3463 children with CF aged <6 years, using the CF Foundation Patient Registry.	10 µg/m^3 increase in $PM_{2.5}$ exposure increased the risk of MRSA acquisition during follow up (Hazard Ratio: 1.68; 95% CI 1.24-2.27).
Kunzi *et al*., 2015 [169]	*In vitro* study assessing the response of normal, distressed (antibiotic treated), CF human bronchial epithelia (HBE), and single-cell bronchial epithelial cell line (BEAS-2B), when exposed to gasoline-exhaust particles.	Short-term exposure to gasoline-exhaust particles impaired epithelial defence mechanisms; promoting cell death in a dose-dependent manner in normal and diseased epithelia. Decreased cytokine release was seen in CF epithelia, thereby lowering the capacity to respond to insult.
Jeannet *et al*., 2017 [170]	*In vitro* study using normal and CF HBE and BEAS-2B cell lines, looking at cell death, inflammatory response, epithelial function and morphology, following exposure to silver and carbon nanoparticles.	Nanoparticle exposure led to increased necrosis in CF compared to normal HBE and BEAS-2B cells; with no functional and structural alterations to the epithelia.
Ni *et al*., 2015 [210]	*In vitro* study using murine macrophages exposed to cigarette smoke extract and *pseudomonas aeruginosa.*	Cigarette smoke extract exposure inhibited *Pseudomonas aeruginosa* phagocytosis, which was improved by induction of macrophage CFTR expression.
Psoter *et al*., 2015 [164]	Retrospective study of 3575 children aged <5 years, using the US registry.	10 µg/m^3 increase in $PM_{2.5}$ exposure was associated with earlier *Pseudomonas aeruginosa* acquisition – a 24% increased risk of Pseudomonas acquisition during follow up.
Geiser *et al*., 2014[167]	*In vitro* study using alveolar epithelial cells from CFTR mutant mice, exposed to iridium, titanium doxide, and carbon nanoparticles.	CFTR mutant epithelial cells showed higher nanoparticle uptake.
Farhat *et al*., 2013 [211]	1 year prospective study of 103 children with CF.	An interquartile range increase (45.62 µg/m^3) in the ozone concentration had an

		OR of 1.86 (95% CI 1.14-3.02) for a 2-day delayed pulmonary exacerbation.
Jassal *et al*., 2013 [212]	5 year retrospective study of 145 patients with CF aged >7 years, using the US Cystic Fibrosis Foundation National Patient Registry.	Correlation between pulmonary exacerbations and 1000 m distance to major arterial roads, with an OR of 6.7 (1.23–54.9).
Goeminne *et al.,* 2013 [163]	Case crossover analysis of 215 patients with CF.	Increasing concentrations of PM_{10}, NO_2, and ozone on the event day and for NO_2 on the day before were associated with increased risk of antibiotic use. OR for pulmonary exacerbation per 10μg/m^3 increase in each pollutant: PM_{10} = 1.043 (1.004–1.084) NO_2 = 1.106 (1.05–1.166) O_3 = 1.034 (1.003–1.067)
Kasmdar *et al*., 2008 [213]	*In vitro* study using CF HBE exposed to $PM_{2.5}$.	Oxidative stress and mitochondrial signalling mediated apoptosis were enhanced by PM, which upregulated pro-apoptotic mediators; and had no effect on anti-apoptotic mediators.
Goss *et al.,* 2004 [166]	Retrospective cohort study of 11484 patients with CF aged >6 years, using the Cystic Fibrosis Foundation National Patient Registry.	10 μg/m^3 rise in PM_{10} and $PM_{2.5}$ was associated with an 8% (95% CI, 2-15%) and 21% (95% CI, 7-33%) increase in the odds of two or more exacerbations, respectively. 10 ppb rise in ozone was associated with a 10% (95% CI, 3-17%) increase in odds of two or more exacerbations. For every 10 μg/m^3 increase in $PM_{2.5}$, there was an associated fall in FEV_1 of 24 ml (95% CI 7-40).

Appendix 4 – Protocol: Reducing the effects of air pollution in children with cystic fibrosis

1. General Information

Full title:	Reducing the effects of air pollution on children with cystic fibrosis
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2. Glossary of Terms and Abbreviations

AE	Adverse Event
AM	Airway Macrophage
AR	Adverse Reaction
ASR	Annual Safety Report
BAL	Broncho-alveolar lavage
BC	Black Carbon
CA	Competent Authority
CF	Cystic Fibrosis
CI	Chief Investigator
COX	Cyclooxygenase
CRF	Case Report Form
CRO	Contract Research Organisation
DMC	Data Monitoring Committee
EC	European Commission
FEV1	Forced Expiratory Volume in 1 second
GAfREC	Governance Arrangements for NHS Research Ethics Committees
GPS	Global Positioning System
ICF	Informed Consent Form
IL-9	Interleukin-8
IS	Induced Sputum
ISS	Induced sputum supernatant
JRMO	Joint Research Management Office
JRO	Joint Research and Development Office
NHS REC	National Health Service Research Ethics Committee
NHS R&D	National Health Service Research & Development
NO_2	Nitrogen Dioxide
Participant	An individual who takes part in a clinical trial or study
PGE_2	Prostaglandin E_2
PI	Principle Investigator
PIS	Participant Information Sheet
PM	Particulate Matter
QA	Quality Assurance
QC	Quality Control
RCT	Randomised Controlled Trial
REC	Research Ethics Committee
SAR	Serious Adverse Reaction
SAE	Serious Adverse Event
SDV	Source Document Verification
SOP	Standard Operating Procedure
SSA	Site Specific Assessment
TMG	Trial Management Group
TSC	Trial Steering Committee

3. SIGNATURE PAGE

Chief Investigator Agreement
The clinical study as detailed within this research protocol **(Version 1.11, dated 19/02/2019)**, or any subsequent amendments will be conducted in accordance with the Research Governance Framework for Health & Social Care (2005), the World Medical Association Declaration of Helsinki (1996) and the current applicable regulatory requirements and any subsequent amendments of the appropriate regulations.

Chief Investigator Name: Professor Jonathan Grigg

Chief Investigator Site: Blizard Institute

Signature and Date: **19/02/2019**

Principal Investigator Agreement *(if different from Chief investigator)*
The clinical study as detailed within this research protocol **(Version 1.11, dated 19/02/2019)**, or any subsequent amendments will be conducted in accordance with the Research Governance Framework for Health & Social Care (2005), the World Medical Association Declaration of Helsinki (1996) and the current applicable regulatory requirements and any subsequent amendments of the appropriate regulations.

Principal Investigator Name: Dr Norrice Liu

Principal Investigator Site: Blizard Institute

Signature and Date: **19/02/2019**

4. Summary / Synopsis

Short title	Air pollution and Children with Cystic Fibrosis
Methodology	Cross-sectional cohort study
Research Sites	• Barts Health NHS Trust • Blizard Institute, Queen Mary University of London • Alder Hey Children's Hospital, Liverpool
Objectives/ Aims	1. To determine personal exposure to air pollution in children with cystic fibrosis; 2. To determine airway macrophage uptake of inhaled particulate matter in cystic fibrosis children; 3. To establish whether prostaglandin E2 affects particulate matter removal in airway.
Number of Participants	Approximately 100
Main Inclusion Criteria	• Age 1-17 • Diagnosis of Cystic Fibrosis • Diagnosis of non-CF bronchiectasis • Living in or around London or Liverpool For sputum induction: with age matched healthy controls For bronchoscopy: controls will be children under investigation for non-inflammatory non-infective respiratory conditions
Statistical Methodology and Analysis	The study is powered to detect a difference of 0.7 SD in macrophage black carbon levels and PGE2 metabolites level between children with CF or non-CF bronchiectasis and control groups.
Proposed Start Date	13/09/2016
Proposed End Date	12/09/2020
Study Duration	4 years

5. Introduction

Background:
Cystic fibrosis (CF) is the commonest hereditary life-shortening respiratory condition, resulting in premature death secondary to recurrent airway infections and inflammation, leading to irreversible lung damage. The screening programme in infancy has improved life expectancy but the lifespan of CF patients is still reduced by 20 years at present [214]. Thus interventions to reduce inflammation, infection and lung damage in CF are still needed [215].

One potential intervention is to reduce exposure of CF children to air pollution. Particulate matter (PM) are microscopic soot particles from petrol and diesel engine emissions; PM is linked to adverse respiratory health effects in children. Children with cystic fibrosis are particularly vulnerable to PM but, to date, no exposure-reduction advice is available, and the mechanism underlying this vulnerability is unclear.

Personal exposure of CF children to PM is due to: i) locally-generated sources (relative to the proximity to busy roads) and ii) background concentrations (i.e. PM blown across the whole city from other areas). Although a link between air pollution and reduced lung function growth in healthy children is well established [61], the link with CF lung disease has only recently emerged. A recent study found that long-term exposure of CF children to PM increases risk of airway infection with *Pseudomonas* [164]. Another study also found that short-term background pollution is associated with increased need for antibiotics in children and adults with CF [163]. The need to reduce patients' personal exposure to PM has been recognised by Barts Health NHS Trust. Working with Global Action Plan, the Trust has developed tips on how to reduce adults' personal exposure to air pollution. These tips include traveling outside rush hours, taking low pollution routes (maps provided), and signing up to the airText air pollution warning App. Since travel outside rush hours is not feasible for school children, child-friendly tips are urgently needed.
Recent research in asthma patients suggests a putative mechanism of impaired removal of inhaled PM. In the healthy lung, inhaled PM is quickly removed (phagocytosed) by airway macrophages (AM). AM patrol the surface of epithelial cells lining the airways; after taking up PM, they move up and out of the lung. Thus AM with normal phagocytic function act to both reduce PM exposure of other airway cells – including epithelial cells, and to ensure that PM does not accumulate in the lung. The amount of carbon particles in AM was previously used to assess the effects of long-term effects of exposure of healthy children and adults [101,216]. The amount of black carbon (BC) in AM reflects the intrinsic capacity of AM to remove PM (phagocytic capacity). AM carbon is significantly lower in conditions such as severe asthma [217], where AM phagocytic capacity is impaired. Furthermore, a role for prostaglandin E_2 (PGE_2) in mediating impaired phagocytic function in asthma has been identified. First, PGE_2 suppresses AM phagocytosis of urban PM *in vitro*; and second, children with severe asthma had increased urinary PGE_2 metabolites [102]. Shift of inhaled PM away from AM to other cells has the potential to stimulate interleukin-8 (IL-8) release by epithelial cells – a phenomenon observed in mouse, where inhalation of non-inflammatory particles produces significant airway neutrophilia when AM phagocytic function is impaired [218].

We hypothesise that impaired handling of inhaled PM by AM contributes to vulnerability of children with CF to air pollution. Specifically, increased vulnerability to inhaled PM in children with CF is due to PGE_2-mediated impairment of AM phagocytosis. The corollary is that children with CF will have; i) reduced levels of AM carbon *in vivo* (reflecting reduced phagocytosis of inhaled PM *in vivo*), ii) reduced capacity of AM to phagocytose carbon PM *in vitro*, iii) increased metabolites of PGE_2 in the airway and urine, and finally, iv) since PGE_2 production is under the control of the enzyme cyclooxygenase (COX, especially COX-2), CF airway cells have increased COX-2 expression, v) COX inhibitors (e.g. Ibuprofen) should suppress PGE_2 production, theoretically improving AM function.
Non-cystic fibrosis bronchiectasis is a respiratory condition with multiple aetiology such as congenital pathology, immunodeficiency, and primary ciliary dyskinesia. Patients with non-CF bronchiectasis demonstrate similar clinical features to those with CF, patients with either conditions

are managed with similar treatments, we therefore hypothesise that the above also apply to children with non-CF bronchiectasis.
There has been evidence that some organisms are capable of reproducing and residing within airway macrophages without being destroyed by the traditional phagocytic pathway[132]. Often, unwell CF and non-CF bronchiectasis patients are admitted to the hospital for intensive antibiotic treatments but a proportion of their sputum samples yield no growth by routine hospital culture. We therefore propose to explore isolated airway macrophages and identify the organisms within – if any are present.

Clinical Data:
There is evidence to support this hypothesis; i) it was previously reported that phagocytosis of inert latex beads is impaired in AM from CF adults (71 ± 15 % phagocytosis in controls vs. 23 ± 9 % in CF) [123], ii), CF is associated with increased airway PGE_2 [144], and iii) in a pilot study, significantly ($p<0.01$) reduced AM carbon in 6 CF children compared with 6 healthy controls (mean 0 vs. 0.3 ± .05, μm^2 $p<0.01$) was found.

Rationale:
To date, the effect of impaired uptake of PM by AM on other airway cells has not been studied *in vitro*. We hypothesise that in co-cultures of human AM and airway epithelial cells exposed to urban PM, PGE_2-mediated impairment of macrophage phagocytosis increases epithelial release of IL-8. Furthermore, since AM are a major source of airway PGE_2 [219], we speculate that there is increased COX-2 expression in AM from CF children. Thus suppression of AM phagocytic function in CF is an autocrine process; i.e. CF AM release more PGE_2 - which in turn suppresses AM phagocytosis. In this study we will seek evidence for abnormal handling of PM by AM from CF children, and model this using cultures of human airway cells and macrophages.

6. Study Objectives

Primary Objectives:

i. To identify the mechanism for impaired removal of PM by AM in children with CF and non-CF bronchiectasis *in vivo* and *in vitro*- using induced sputum (IS) or broncho-alveolar lavage (BAL) fluid following diagnostic bronchoscopy, compared to healthy controls or those who are undergoing bronchoscopy to investigate for non-inflammatory non-infective conditions (e.g. those who do not have CF, non-CF bronchiectasis, or asthma).
ii. To test the hypothesis of PGE_2 involvement in AM phagocytosis of PM - by comparing PGE_2 metabolites in respiratory secretions and urine of children with CF and non-CF bronchiectasis (both during periods of stable health status and exacerbations) to controls.
iii. To model whether decreased uptake of PM by macrophages *in vitro* increases release of cytokines (e.g. IL-8) from airway epithelial cells *in vitro*.

Secondary Objectives:

i. To identify the major sources of personal exposure of children with CF and non-CF bronchiectasis to air pollution – this will be compared to healthy controls.
ii. To determine the amount of diesel soot in AM from children with CF and non-CF bronchiectasis and healthy controls.
iii. To develop feasible, acceptable and evidence-based advice on short and long-term exposure reduction.
iv. To explore and identify intracellular bacteria strains, if any, from within airway macrophages, and compare with routine hospital culture results.
v. To explore whether COX inhibitors can reduce PGE_2 production.

Endpoint:

i. Comparing personal exposure of children with CF and non-CF bronchiectasis to air pollution to healthy controls.
ii. Quantifying the amount of black carbon in AM from children with CF and non-CF bronchiectasis and healthy children.
iii. Quantifying and comparing PGE_2 metabolites in respiratory secretions and urine of children with CF and non-CF bronchiectasis and healthy children.
iv. Measuring cytokines (e.g. IL-8) from airway cells of children with CF and non-CF bronchiectasis and healthy controls.

7. Methodology

Inclusion criteria:

- Children with Cystic Fibrosis with age specified as above
- Children with non-CF bronchiectasis with age specified as above
- For children undergoing sputum induction: Age-matched healthy controls
- For children undergoing bronchoscopy: controls will be children without CF, non-CF bronchiectasis or other inflammatory or infective respiratory conditions (e.g. those undergoing bronchoscopy for investigation of airway anatomy but are otherwise well).
- All children should reside in urban areas.

Exclusion criteria:

- Current active smoker
- Receiving immunosuppressive drug therapy
- For children with CF and non-CF bronchiectasis not on regular nebulised hypertonic saline: drop in FEV1 of >15% post-bronchodilator (exclusion criterion for sputum induction) – this only applies to those undergoing sputum induction.
- For healthy controls: Post-bronchodilator FEV1 <80% (standard exclusion criterion for sputum induction in healthy individuals) – this only applies to those undergoing sputum induction.
- For participants currently or recently involved in other research study, they will be excluded if their current or recent research have any potential impact on our sampling or results.

Study Design:

Cross sectional cohort study of children with CF and non-CF bronchiectasis recruited in London and Liverpool. CF and non-CF bronchiectasis children will be recruited at the participating hospital sites; controls (those without inflammatory respiratory conditions) will be recruited from children living in urban areas in London or Liverpool. In view of the CI's particular interests and expertise in air pollution, and his contacts within the British media, charities, and health and education sectors, healthy children across London may also be invited to take part through these organisations.
Potential participants will be identified and approached in the first instance by a member of their usual clinical care team. The research team will then review and ensure they meet the eligibility criteria.

The study is planned for the duration of 4 years. Participants' will actively participate (monitoring and sampling) for an overall of 2 years, except for those undergoing bronchoscopy – their participation will end after bronchoscopy.

Recruitment:

We aim to recruit approximately 100 children.

Personal Exposure Study:

We will monitor personal exposure of all recruited children including:

- Approximately 40 children with CF and non-CF bronchiectasis (age 7-17)

- Approximately 40 healthy controls (age 7-17)
- Approximately 10 children with CF and non-CF bronchiectasis (age 1-6)
- Approximately 10 healthy controls (age 1-6)

Unless the children are undergoing bronchoscopy, they will only take part in the exposure monitoring and urine collection parts of the study. (Sputum induction is difficult under the age of 7)

Induced Sputum:
Approximately 40 children with CF and non-CF bronchiectasis (age 7-17) and approximately 40 age-matched controls.

Bronchoscopy (performed for diagnostic reasons not related to this project):
Approximately 10 children with CF and approximately 10 controls without CF/ non- CF bronchiectasis.

Urine collection:
All children as above – inability to produce a urine sample will not exclude participation.

8. Study Procedures

Patient information sheets will be given out to potential participants. Informed consent and assent will be gained from participants and their parents/guardians during the recruitment phase of the study, after dissemination of information sheets.

Data will be stored in paper and electronic formats. Participants' data will be collected in Case Report Form which will be stored within the investigator's site files, securely located in a locked cabinet within the Blizard Institute. Data stored on university computers will be de-identified.

Only participants' identification number will be recorded in the Case Report Form. The only document linking their personal details (name) to their participant ID is the consent form - one copy will be given to the patient, one copy to be stored in medical records and one copy to be kept within the investigator's site file. For Audit purposes, Sponsors/monitors will need to view information.

Schedule of Assessment:

Personal exposure
With informed consent, we will record children with CF and non-CF bronchiectasis and controls' demographics (postcodes, contact details), medical history, +/- usual travel routes, and sources of indoor and outdoor air pollution exposure (e.g. cigarette smoke, cooking) in the Case Report Form, for the purpose of pollution exposure monitoring, and potential house visit to carry out lung function and sputum induction.

For children recruited in London, a black carbon PM monitor will be carried by each participant for 2 x 24 hour periods. A parent/child completed activity diary will identify routes and activities associated with high exposure. Nitrogen Dioxide (NO_2) is a valid marker for fossil-fuel derived PM (outdoor) and natural gas e.g. cooking gas and heating (indoor). 2-week cumulative exposure will be assessed using NO_2 badges. One NO_2 badge will be placed near the child (e.g. worn by child/carer, attached to buggy), an additional NO_2 badge will be placed inside the participant's home to detect indoor gas cooking emissions (a PM- independent source). Current health status of children with CF and non-CF bronchiectasis (e.g. lung function, medications, infection status) will be obtained from clinical records. The results will compare air pollution exposure of children with CF and non-CF bronchiectasis to healthy controls.

Mechanism for impaired removal of PM

We will compare *in vivo* AM carbon in children with CF and non-CF bronchiectasis and healthy age-matched controls by sputum induction or following diagnostic bronchoscopy. Sputum induction will only be done in children > 7years of age for practicality reasons. The induced sputum samples will then be used for AM black carbon analysis. For children who are undergoing bronchoscopy for diagnostic reasons (not related to this research project), any residual bronchoalveolar lavage (BAL) will also be used for research purpose.

- **Spirometry (Lung Function) – for those undergoing sputum induction only**
  Lung function will only be done in children over the age of 7 for practical reasons. After baseline lung function, participants will be given 400μg salbutamol by metered dose inhaler and spacer. "Post-bronchodilator" lung function will be obtained after 15 min. Forced expiratory volume in 1 sec (FEV_1), forced vital capacity (FVC), FEV_1/FVC and mid-expiratory flow between 25% and 75% of the forced vital capacity (FEF_{25-75}) will be determined. A further lung function will be repeated following sputum induction to ensure no side effect, which may require treatment, has occurred.

- **Sputum Induction**
  This will be done in all recruited children over the age of 7 for practical reasons, the process will take approximately 30 minutes. Sputum (containing lower airway cells including airway macrophages) will be induced using nebulised 3.5-7% saline and an ultrasonic nebuliser. Participants will be given Salbutamol prior to nebulised hypertonic saline to limit the effect of bronchospasms, and lung function will be monitored (see above). Clinical samples are brought to the Blizard Institute for analysis within 4 hours. Slides will be analysed using our established methodology.

  For children with CF and non-CF bronchiectasis who regularly use nebulised hypertonic saline, their routine practice will be replaced by our standardised protocol, including Salbutamol use and lung function monitoring. Sputum induction can be done in a place most convenient to the child/family (e.g. hospital, home, school). The timing of sputum induction can be synchronised with children's (those with CF and non-CF bronchiectasis) routine chest physiotherapy session to limit inconvenience. We may obtain multiple induced sputum samples (limiting to 1 sample/ day) – sputum induction is similar to these children's daily chest physiotherapy, therefore obtaining multiple samples should be feasible, but this will be guided by children's co-operation.

  For healthy controls, we will limit the number of samples to a maximum of 3. Sputum induction will be done at the Royal London Hospital, or at an appropriate and safe place which is convenient for the participants and their family – such as home or school, with close monitoring of lung function (see above). Our research team has vast experience on the procedure and have performed spirometry and sputum induction on over 400 children in their schools, with no major complications. Researchers who perform the procedures outside the hospital are trained paediatric doctors and are able to provide medical attention when needed, although this is not anticipated to be necessary.

Bronchoscopy
For children undergoing diagnostic bronchoscopy, the BAL samples collected will be residual of that used for diagnostic purpose – to analyse AM function and AM black carbon analysis.

The induced sputum samples will be processed in the Blizard Institute, and used for AM function analysis. The BAL samples will be processed in the local laboratory (London or Liverpool) and used to assess AM function. Following purification process of AM, they will also be used to model the effect of impaired AM phagocytosis of PM. Presence of Cyclooxygenase (COX)-2 will be quantified. Part of the sputum samples will be used for bacterial culture. Respiratory secretions supernatant and urine samples (minimum of 1ml) will be anonymously labelled and transported to the analysis laboratory (in Poland) for prostanoid metabolites analysis. Participants undergoing sputum induction will be given the option to take a routine over-the-counter (no prescription needed) course and dose of COX- inhibitor (for example, a 3 day course of Ibuprofen – see below), followed by repeat measurements of PGE2 metabolites in their IS supernatant and urine samples.

Feedback of findings to parents / children – London only
For the participants in London, the above data, including individual black carbon PM findings, will be analysed and discussed with the children and their families. Options for reducing outdoor and indoor exposure will be discussed (e.g. alternative routes when travelling to school, improved ventilation during gas cooking). If appropriate, maps will be generated using an urban walking route planner and/or http://www.londonair.org.uk/london/asp/annualmaps.asp to show alternative routes to minimise exposure. The feasibility of adapting the adult-orientated Barts Health tips and exposure maps, and relevance of advice from airText will also be assessed.

Should any incidental findings for healthy controls, relevant and significant to their health, be identified, the researchers will contact the participants as soon as possible and advise regarding management.

Co-design of evidence-based guidance/tips for clinical use – London only
After completion of data analysis, and in collaboration with the Trust, we will arrange further parent engagement meetings to co-design evidence-based Trust-wide exposure reduction guidance/tips for children with CF and non-CF bronchiectasis, with the participants in London.

Assessment	Recruitment phase	Initial visit	Study phase	End of study
Dissemination of information leaflets	**x**			
Informed consent	**x**			
Case Report Form		**x**	**x**	
Black carbon monitoring		**x**	**x**	
Nitrogen Dioxide monitoring		**x**	**x**	
Spirometry		**x**	**x**	
Sputum Induction		**x**	**x**	
Urine collection		**x**	**x**	
Parent engagement meetings				**x**

End of Study Definition

End of study will be when data collection is complete. Participants' participation will complete before data analysis is finished - participation will involve air pollution monitoring (London), sampling (London / Liverpool) and engagement meeting (London) near the end of study. It is not anticipated there will be premature termination of the study. Should any subject experience a serious adverse event associated with or attributable to any study procedure, the study status will then be reviewed.

9. Statistical Considerations

We aim to recruit 40 school age children with CF or non-CF bronchiectasis and 40 age-matched controls. 40 children per group will provide power of 90% at 5% to detect a difference between groups of 0.7 SD. Cell culture data will be analysed by either one-way analysis of variance and Tukey's *post hoc* test for > 2 groups, or unpaired t test for 2 groups.

Additional observational personal exposure data and urine samples will be obtained from 10 children with CF and non-CF bronchiectasis of 1-7 years, with 10 age-matched controls - the maximum feasible number in a 3-year time frame.

10. Ethics

Ethical Review

The protocol will be reviewed both within the Sponsor Institution and by an independent research ethics committee. All the techniques are ethically acceptable in children.

Rationale for research

Ethical research must be informed by existing research, and investigate an important question. We have addressed this issue by thorough review of the existing literature, and by conducting appropriate background studies.

Design of research

Ethical research must employ the most appropriate design in order to answer the research question. A cross-sectional cohort study design has been chosen to address the hypothesis.

Minimisation of inconvenience, discomfort and risk for participants

Ethical research must seek to minimize potential inconvenience, discomfort and risk that participants may experience during the course of a study. The principle inconveniences of the study to the participants are minimized through use of qualified and trained practitioners to collect sputum and urine samples. Any participants who are undergoing bronchoscopy are doing so for diagnostic reasons, no additional procedure will be required to take part in this study. Salbutamol and Hypertonic Saline will be applied according to British National Formulary recommendations. Inconvenience is further reduced by performing sampling in the most appropriate and convenient time and place for child/family. We have extensive expertise in modelling environmental interactions with airway cells *in vitro*, immunostaining of IS cells (in an on-going MRC-funded study of dendritic cells), and in personal monitoring. Regarding COX inhibitor (e.g. Ibuprofen), this is a commonly used medication for fever and pain, with most of the general population having been exposed to the use of this medication since infancy – a lot of the participants will have used this medication previously – medical staff on the research team will ensure there is no known allergy or contra-indications to this medication before offering this to participants.

11. Safety Considerations

Risks

i. Sputum Induction

Sputum induction with ultrasonic nebulisation of hypertonic saline is a procedure that is used therapeutically in children with CF and non-CF bronchiectasis. The procedure is well-tolerated, and we have extensive expertise in performing induced sputum in healthy children (over 400 children studied to date). The small risk of bronchospasm is significantly reduced by pre-treatment with inhaled salbutamol. Inhalation of hypertonic saline can cause a sore throat, however the effect is mild and short-lived.

ii. Lung Function

Lung function is a safe, non-invasive procedure and will be performed according to standard European Respiratory Society Guidelines.

iii. Urinalysis

Urine samples will be obtained by the participant passing urine into a universal container in privacy, accompanied by a responsible adult if required.

iv. Carbon monitoring

Carbon monitoring via use of a small, lightweight portable meter is convenient and non-invasive. The NO_2 badges will be placed near the child during the 2 weeks; for young children, this can be attached to their buggy/carer to avoid accidental ingestion hazard; or it can be attached to older children's clothes.

v. Bronchoscopy

Flexible Bronchoscopy is being done for existing clinical reasons. The procedure will never be done solely for the purpose of this study. The study will merely make use of any residual diagnostic samples for research.

vi. Investigator safety

Investigators will prevent personal exposure to airborne or blood-borne infectious organisms by appropriate use of masks, goggles, lab coats and gloves while obtaining and processing samples. Lone worker policies (NHS and QMUL) will be in place for investigators to carry out home visits.

Benefits

Participating children and families will benefit from increased knowledge and understanding of air pollution in their environment and ways to try decrease their exposure. We will produce feasible and evidence-based guidance to reduce pollution exposure – this has immediate applicability in our CF and Bronchiectasis clinics. By establishing biological plausibility for the interaction between air pollution and lung disease in children with CF and non-CF bronchiectasis, this study will strengthen the case for causality and thereby support exposure-reduction intervention policy. We will provide clinicians with the tools to answer parent's questions about air pollution, in order to enhance patient experience. The acceptability of the guidance is improved by inclusion of patient/parent in its design.

12. Data Handling and Record Keeping

Confidentiality

Ethical research projects should ensure that participants' personal data remain confidential. Our procedures for handling, processing, storage and destruction of data

are compliant with the Data Protection Act 2018. Participants will be allocated a participant ID which will be used as a code to identify them on all study forms and samples. The only document that will link the participant's personal details with the participant ID is the consent form, which will have 3 copies – one to be given to the patient, one to be kept in their medical records, one to be kept in the investigator's site files which will be locked in a cabinet within the Blizard institute, only accessed by research team. Data will be stored in paper and electronic formats. Data stored on university computers will be de-identified. For Audit purposes, Sponsors/monitors will need to view information.

Record Retention and Archiving
In line with Good Clinical Practice guidelines, at the end of the study, your data will be securely archived for a minimum of 20 years. Arrangements for confidential destruction will then be made.

13. Laboratories

A) Blizard Institute, London

Lab Procedures:

i. **AM Black Carbon Analysis**
Cytospins stained by Diff-Quik are analysed for AM black carbon using digital colour images of 50 randomly selected AM from each child, captured at x100 magnification [101]. The total area of carbon in each AM is calculated by measuring the black content of each cell. Mean AM carbon per child is then calculated from the 50 AM. To ensure exposure to fossil-fuel derived air pollution is similar between the CF, non-CF bronchiectasis and controls, we will compare their personal 2-week NO_2 exposure. We will also record the distance between home/ school and nearest main road, and mean annual exposure to PM_{10} from the nearest monitoring station to the home.

ii. **Relationship between impaired PM uptake and cytokines release**
AM sampled by IS will be purified (25), incubated with ultrafine carbon black *in vitro* for 2 hours at a concentration resulting in near maximal loading of control AM. Uptake of ultrafine carbon between CF, non-CF bronchiectasis and controls will be compared, after adjusting for *in vivo* basal loading. In children (CF, non-CF bronchiectasis and controls) who produce large numbers of AM, and/or are willing to undergo repeated IS, we will also assess non-specific phagocytosis of latex beads by AM using our flow cytometry assay [123]. Cells will be cultured with urban PM_{10}, with and without exogenous PGE_2 using our standard method [220,221]. In this co-culture system, normal macrophage phagocytic function results in all PM being taken up by macrophages [222]. IL-8 in co-cultures will be assessed by ELISA.

iii. Presence of Cyclooxygenase (COX)-2 in AM will be quantified.

iv. Purified AM from sputum samples will be cultured to identify, if any, intracellular organisms – the results will then be compared to routine hospital sputum culture.

Data Preparation and Collection:
Samples will be labelled with the participant ID given to each participant – samples will be pseudo-anonymised. Date of collection and conditions at which the samples are sent and storied will be recorded to ensure integrity. Samples are collected from the

Royal London Hospital or participant's home, then sent to the Blizard institute for analysis within 4 hours. For procedures that do not have to be performed on the same day, the samples will be securely stored within the Blizard Institute.
Following analysis, if any samples remain, 1ml aliquot of sputum and urine samples will be stored at -80C in a freezer located within the research institute – in case of any need for repeated experiments for the study. The remainder of the samples will be discarded according to our local disposal policy.

B) Alder Hey Children's Hospital, University of Liverpool

Lab Procedures:
Residual from routine Bronchoalveolar Lavage (BAL) samples will be processed for assessment of AM function. This will involve producing a cytospin microscopy slide from approximately 1ml of BAL fluid. The slide will be stained and transported to QMUL. While the cell pellet will be used for cytospin as above, the supernatant will be frozen (-80C) and transported to QMUL for functional analysis and prostanoid quantification.

Urine (at least 1ml) from participants will be frozen (-80C) after collection prior to transport to QMUL. Inability to collect urine will not exclude participation.

Data Preparation and Collection:
Samples will be labelled with the participant ID given to each participants, along with sampling date – samples will be pseudo-anonymised. Date of collection and conditions at which the samples are sent and storied will be recorded to ensure integrity. Samples are collected and processed at Alder Hey Children's Hospital, then sent to the Blizard institute for further processing and analysis.

Following processing, if any samples remain, aliquots of BAL samples will be stored at -80C in a freezer located within the research institute – in case of any need for repeated experiments for the study. The remainder of the samples will be discarded according to local disposal policy.

C) Department of Medicine, Jagiellonian University Medical School, Krakow, Poland

Lab Procedures:
Total ISS protein concentration is measured (mg/mL). The concentration of PGE_2 metabolites in ISS is measured by high-performance liquid chromatography/tandem mass spectrometry using organic phase extraction [223]. These metabolites of PGE_2 will also be assessed in participants' urine [102]. Metabolites of PGE_2 in respiratory secretions and urine samples from children with CF or non-CF bronchiectasis and controls will be compared.

Data Preparation and Collection:
Samples will be labelled with the participant ID given to each participant – samples will be pseudo-anonymised. Date of collection and conditions at which the samples are sent and storied will be recorded to ensure integrity. Samples are collected from the Royal London Hospital or participant's home, then stored at the Blizard institute. IS supernatant (ISS) is then transported to the analysis laboratory. Samples will be discarded following analysis. Analysis results will be sent to the Blizard in an pseudo-anonymised form, using only participant IDs.

14. Products, Devices, Techniques and Tools

Magee Scientific portable Aethalometer

Devices:

Carbon Monitoring
We will measure 24-hour personal external black carbon exposure in each child using a portable Aethalometer. Children carry the monitor in a small bag for 24 hours alongside a time/activity diary, which is filled in by child/parent. These monitors give a real time picture of exposure to pollution. A GPS device will identify their routes of traveling. We will measure 24-hour exposure on two separate occasions.

Pollution Monitoring

Nitrogen Dioxide Monitoring
A NO_2 diffusion monitor (the size of a small badge) will be placed near child for 2 weeks to monitor their NO_2 exposure. This can be placed on the buggy/ parent/ carer or on child's clothes, depending on their age and level of cooperation. An additional NO_2 badge will be placed inside the participant's home to detect indoor natural gas exposure. The data will be analysed by the manufacturers of the NO_2 diffusion monitor (IVL Swedish Environmental Research Institute Ltd, P.O. Box 53021, SE-400 14 Gothenburg, Sweden) (http://www.diffusivesampling.ivl.se/oursamplers.4.75d7780712240e747ea80004619.html)

All devices are already owned by the research team at the Blizard Institute so no purchase is required.

Techniques and Tools:

Lung function
This will be measured using MicroMedical MicroLab 3500 Spirometer Mk 8 with a data management system compliant with ATS/ERS 2005 guidelines. Flow volume loops are displayed for immediate quality control and stored for export to a relational database using customised software. Both unadjusted lung function and % predicted values adjusted for age, gender and height will be shown, together with variables describing adherence to standardised measures of technical quality such as start of test and end of test acceptability criteria and forced expiratory time.

Medicinal products:

Salbutamol – short-acting β2 agonist which comes in a pressurised inhaler.
Indication: Bronchodilator used for reversal of airway obstruction (e.g Asthma, wheeze).
Licensed to be used in participants' age group in the UK.
Dose/ Route: 400 micrograms (4 puffs) via inhaler.
Storage: stored in room temperature.
Side effects: fine tremor, nervous tension, headache, muscle cramps, and palpitation. Other side-effects include tachycardia, arrhythmias, peripheral vasodilation, myocardial ischaemia, and disturbances of sleep and behaviour. Paradoxical bronchospasm (occasionally severe), urticaria, angioedema, hypotension, and collapse have also been reported

Hypertonic Saline (3.5-7%)
Indication: mobilise lower respiratory tract secretions in mucous consolidation (e.g. CF)
Licensed to be used in participants' age group in the UK.
Dose/ Route: 4ml via nebuliser.

Storage: stored in room temperature.
Side effects: Bronchospasm

Cyclo-oxygenase inhibitor (Ibuprofen)
Indication: Mild to moderate pain; pain and inflammation of soft tissue injuries; pyrexia with discomfort.
Licensed to be used in participants' age group in the UK.
Dose/ Route: as per British National Formulary (BNF)

- Child 7-9 years: 200mg 3 times a day, by mouth
- Child 10-11 years: 300mg 3 times a day, by mouth
- Child 12-17 years: 400mg 3 times a day, by mouth

Storage: stored in room temperature.
Side effects:

General side-effects

Uncommon
Gastrointestinal discomfort; hypersensitivity; rash (discontinue); skin reactions

Rare or very rare
Angioedema; dyspnoea

Frequency not known
Asthma

Specific side-effects

Uncommon
Headache; nausea

Rare or very rare
acute kidney injury; agranulocytosis; anaemia; constipation; diarrhoea; fatigue; fever;
gastrointestinal disorders; haemorrhage; hypotension; influenza like illness; leucopenia; liver disorder; meningitis aseptic (patients with connective-tissue disorders such as systemic lupus erythematosus may be especially susceptible); oedema; oral disorders; pancytopenia; renal papillary necrosis; respiratory disorders; severe cutaneous adverse reactions (SCARs); shock; tachycardia; throat pain; thrombocytopenia; vomiting

Frequency not known
Crohn's disease; fertility decreased female; fluid retention; heart failure; hypertension; increased risk of arterial thromboembolism; renal failure (more common in patients with pre-existing renal impairment); respiratory tract reaction.

15. Safety Reporting

Adverse Events (AE)
An AE is any untoward medical occurrence in a subject to whom a medicinal product has been administered, including occurrences which are not necessarily caused by or related to that product. An AE can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding), symptom or disease temporarily associated with study activities.
Notification and reporting Adverse Events or Reactions
If the AE is not defined as SERIOUS, the AE is recorded in the study file and the participant is followed up by the research team. The AE is documented in the participants' medical notes (where appropriate) and the CRF.

Serious Adverse Event (SAE)
This is defined as an untoward occurrence that:
(a) results in death;
(b) is life-threatening;
(c) requires hospitalisation or prolongation of existing hospitalisation;
(d) results in persistent or significant disability or incapacity;
(e) consists of a congenital anomaly or birth defect; or
(f) is otherwise considered medically significant by the investigator.

An SAE occurring to a research participant should be reported to the main REC where in the opinion of the Chief Investigator the event was:
• Related – that is, it resulted from administration of any of the research procedures, and
• Unexpected – that is, the type of event is not listed in the protocol as an expected occurrence.

Notification and Reporting of Serious Adverse Events
Serious Adverse Event (SAEs) that are considered to be 'related' and 'unexpected' are to be reported to the sponsor within 24 hours of learning of the event and to the Main REC within 15 days in line with the required timeframe. Refer to NRES website and JRMO SOPs for further guidance.

Urgent Safety Measures
The CI may take urgent safety measures to ensure the safety and protection of the subjects from any immediate hazard to their health and safety. The measures should be taken immediately. In this instance, the approval of the REC prior to implementing these safety measures is not required. However, it is the responsibility of the CI to inform the sponsor and Main Research Ethics Committee (via telephone) of this event immediately.
The CI has an obligation to inform both the Main REC in writing within 3 days, in the form of a substantial amendment. The sponsor (Joint Research Management Office [JRMO]) must be sent a copy of the correspondence with regards to this matter. Refer to NRES website and JRMO SOPs for further guidance.

Annual Safety Reporting
The CI will send the Annual Progress Report to the main REC using the NRES template (the anniversary date is the date on the MREC "favourable opinion" letter from the MREC) and to the sponsor. Please see NRES website and JRMO SOP for further information

Overview of the Safety Reporting responsibilities
The CI/PI has the overall pharmacovigilance oversight responsibility. The CI/PI has a duty to ensure that safety monitoring and reporting is conducted in accordance with the sponsor's requirements.

16. Monitoring and Auditing

A data monitoring committee will not be convened. Intermittent random audit of data quality will be performed by members of the investigating team under the supervision of the Chief investigator (CI).

Research sponsor will ensure arrangements and systems are in place for the management and monitoring of research.

The arrangements will be in accordance to the Research Governance Framework.

17. Trial Committees

This study is not a clinical trial – a trial committee is therefore not required. However there will be a study committee consisting of the Chief Investigator and representatives from the Research and Development Department – they will meet quarterly to ensure the study is progressing satisfactorily.

18. Finance and Funding

This study is funded by Barts Charity (grant reference: MGU0312).

19. Indemnity

This study will be sponsored by Queen Mary University of London. Contact details as previously stated. **Queen Mary University of London** will arrange for suitable indemnity for negligent harm arising as a result of participation in this study to be in place. The protocol will be evaluated by the Governance Officer, Queen Mary University of London.

20. Dissemination of Research Findings:

Any manuscript reporting study findings will be prepared according to CONSORT guidelines and submitted to peer-reviewed biomedical journals according to ICMJE Uniform Requirements. Authorship will be based on individuals' contribution to study design, conduct, analysis, drafting/revision of manuscript and final approval of the version to be published. Authorship will not necessarily be restricted to individuals named on this protocol; neither is authorship guaranteed to any individual named on this protocol. Contributors who do not meet authorship criteria will be listed in 'Acknowledgements'. The results of the study will be disseminated to the participants and their family in a patient-engagement meeting towards the end of the study.

21. Appendix

Appendix 1 – Black Carbon Monitoring:
https://aethlabs.com/microaeth/ae51/overview

Appendix 2 – Nitrogen Dioxide Monitoring:
http://www.diffusivesampling.ivl.se/oursamplers.4.75d7780712240e747ea80004619.html

Appendix 5 – CF study: Examples of participant information sheets

<CF AGE 16-17> PARTICIPANT INFORMATION SHEET Version 1.7 30/10/2018
REC reference: 17/EM/0023
IRAS number: 215879
Name of researcher: Dr Norrice Liu

PART 1

1. Invitation

You are being invited to take part in a research study. Before you decide whether or not to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully, and discuss it with others if you wish.
PART 1 tells you the purpose of this study and what will happen to you if you take part.
PART 2 gives you more detailed information about the conduct of the study.
Please ask us if there is anything that is not clear, or if you would like more information. Take time to decide whether or not you wish to take part.

2. What is the purpose of the study?

i. This study aims to see how the lung cells in children and young people with Cystic Fibrosis (CF) are affected by the soot from air pollution (e.g. traffic fumes, cooking fumes). We wish to identify children and young people's sources of pollution exposure.
ii. The findings will then be discussed with children/young people and parents. By engaging children/young people and parents, we intend to co-design tips/guidance on reduction of CF children/young people's exposure to air pollution, thereby improving their quality of life.
iii. We will look into whether concentration of certain substances called Prostaglandin (their presence can be detected in phlegm and urine) are associated with clearance of soot in the lungs.
iv. We will find out if reducing the concentration of Prostaglandin (using the medication Ibuprofen) would improve clearance of soot.

3. Why have I been chosen?

You have been asked to take part because you have the condition Cystic Fibrosis, and live in or around London - London is known to have some of the most polluted air in Western Europe.

4. Do I have to take part?

No. It is up to you to decide whether or not to take part. If you decide you would like to take part, then you will be given this information sheet to keep and be asked to sign a consent form, to confirm that you understand what is involved when taking part in this study. If you decide to take part you are free to leave the study at any time and without giving a reason. If you withdraw, unless you object, we will still keep records relating to your participation up to that point, as this is valuable to the study. A decision to withdraw at any time, or a decision not to take part, will not affect the quality of any care you may require from us in the future.

5. What will happen to me if I take part?

If you are interested in taking part, trained researchers will arrange a time to meet you at the Royal London Hospital – this can coincide with your next hospital visit if you have one pending. We will review your medical record for information such as CF status, baseline lung function, medications.

We will then ask you to do the following –

i. We will ask you several questions relating to your potential exposure to air pollutions (e.g. mode of transport to/from school, presence of smokers at home, etc)
ii. **Carrying a small pollution monitor** for 24 hours, on 2 separate occasions, along with a GPS device to map your travel route.
iii. Having 2 small **pollution monitor badges** for 2 weeks – one attached to your clothes to detect outdoors pollution exposure, one being placed at home to detect indoors pollution (e.g. cooking gases).
iv. **Lung function** – This is the same as you normally do in clinic. It involves blowing into a machine to test how strong your breathing is. This should not take more than 20 minutes.
v. **Induced sputum** - Coughing up sputum (mucus and material from the lungs) into a pot following nebulised hypertonic saline (inhaled salty vapour). This will be similar to your routine chest physiotherapy. The sample will then be looked at under a microscope.
vi. Giving a **urine** sample.
vii. Prostaglandin reduction – you will be offered a standard "over-the-counter" course (usually 3 days) and dose of Iburprofen – a medication commonly used for fever or pain; followed by repeat sputum and urine collection.
viii. Attend **patients/parents engagement meetings**.

We anticipate that you will actively be involved in the study for 2 years overall for sampling and monitoring, plus the patients/parents engagement meetings towards the end.

6. What is the procedure that is being tested?

Carrying a pollution monitor is simple – we have small pollution monitors that are about the size of juice carton. They have a hose coming out of them that sucks in air and measures how much soot there is in the air around you. We ask you to carry them on your clothes for 24 hours, on 2 separate occasions, along with a GPS device to map your travel routes, to get an accurate picture of when and where you are exposed to air pollution.

Pollution Monitor

Pollution Monitoring Badge

We will also ask you to attach a small **pollution monitoring badge** to your bag or clothes to monitor the pollution over 2 weeks. This badge is the size on a 10 pence piece and attaches using a small badge pin. There are no special instructions for looking after the badge but it should remain for 2 weeks. If it gets lost, please inform the CF team or the research team. A second badge will be placed at home to detect any indoor pollution exposure (e.g. cigarette smoke, cooking fumes).

We will ask you to perform **lung function** (a test that measures how quickly you can expel air from your lungs). We will then give you four puffs of Salbutamol (blue) inhaler and repeat the lung function test. This reassures us that there is no airway narrowing reactions (called bronchospasm) to the next stage of the induction.

Sputum induction is a way to get a sample of cells from inside the lungs. It is usually well tolerated and safe. This is similar to your routine chest physiotherapy. We will ask you to put the nozzle of a handheld nebuliser in your mouth and inhale the salty vapour it produces. It does not taste unpleasant - it's a bit like standing on a beach near breaking waves – just a bit saltier. After some time this salty vapour will soften up the sputum in the lungs and you will then be asked to spit this out into a container for analysis. You will be able to pause the inhalation to rinse your mouth or blow your nose (it can make your nose run a little) at any point. Most children/ young people complete the procedure well within 30 minutes. We will repeat lung function at appropriate intervals during the study. We will stop the sputum induction at any point that you feel uncomfortable. This may be carried out at a time and place most convenient to you (e.g. to synchronise with your routine nebuliser/chest physiotherapy) – the researcher will discuss the details with you. Depending on the level of success, we may ask you to repeat sputum induction on separate occasions, limiting to 1 sample/day (e.g. baseline state when well, during hospital admission, towards end of hospital admission). The research team will discuss with you regarding the number of times of sputum induction.

Urine sampling involves passing a small amount of urine into a pot. This can be done privately.

Ibuprofen is a medication commonly used for fever or pain and is generally well tolerated in the general population. If there is no allergies or contra-indications, the research doctor will offer you a short standard "over-the-counter" course of Ibuprofen, which will be provided by the research team. The dose and course used will be according to the current licence of the medication and not require any prescription.

Participant/Parent engagement meetings will be held following pollution monitoring data collection and analysis. The findings will be presented to children/young people and parents. We then aim to co-design guidance/tips on air pollution exposure reduction with children/young people and parents.

7. What do I have to do?

If you agree to take part, you can sign a consent form and give it back to any of the CF doctors or nurses. We will then provide you with the pollution monitors and collect samples.

8. What are the alternatives for testing?

It is difficult to otherwise get cells from the lungs – previously it would require an anaesthetic to obtain samples of cells from unwell children/young people in hospital (and obviously we do not want to do that in our air pollution study).

9. What are the side effects of any treatment received when taking part?

Part of testing lung function involves giving a medication that relaxes the muscles in the airways of the lungs – it is called Salbutamol (Ventolin) and comes in a blue inhaler. Children with asthma use salbutamol to help their airways/lungs relax when they get "tight-chested" or "wheezy". We use salbutamol to measure whether air pollution might be making children's airways slightly narrowed, and to help with the sputum induction (see below). Salbutamol is a very safe drug used by thousands of children in the UK. It has mild side effects – it can make the heart beat a little faster for a few minutes after inhaling it. This effect wears off quickly (after 15-30 minutes) as the body uses up the medication. Details on the side effects Ibuprofen can be found in Appendix 1.

10. What are other possible disadvantages and risks of taking part?

Care should be taken when placing the monitoring badge, in order to avoid accidental swallowing hazard.

Sputum induction will take around 30 minutes. There is a very slight risk of wheeze developing when you inhale the salty solution (hypertonic saline), but this is minimised by giving the Salbutamol (blue) inhaler before induction; if wheeze does develop after hypertonic saline, we can treat it very quickly by giving more puffs of Salbutamol. We have done more than 400 sputum inductions in school children and have not had a problem with wheezing, but this is to make you aware that there is a small chance that it could occur. Ibuprofen has been used in children for years, it is a well-tolerated medication and most of the general population will have been exposed to its usage in infancy or childhood. You will only be offered this if there is no allergy or contra-indications, your doctor will go through this in details with you.

Giving a urine sample is quick and easy to do – this can be done in private, or with the help of an adult.

11. What are the possible benefits of taking part?

We hope to show how air pollution affects the airways and lungs of children and young people with Cystic Fibrosis. We wish to co-design guidance/tips in reduction of air pollution exposure with CF children and parents. This study may influence how we try to reduce air pollution exposure and potentially limit its effect on CF children/young people's lungs.

12. What happens when the research study stops?

Your samples will be disposed of and your anonymised data will be analysed and published in a medical journal.

13. What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your question. Our contact details can be found at the bottom of this sheet. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure or contact Patient Advice and Liaison Service (PALS) (details are at the end of this sheet).

Queen Mary University of London has agreed that if you are harmed as a result of your participation in the study, you will be compensated, provided that, on the balance of

probabilities, an injury was caused as a direct result of the intervention or procedures you received during the course of the study. These special compensation arrangements apply where an injury is caused to you that would not have occurred if you were not in the study. These arrangements do not affect your right to pursue a claim through legal action.

14. Will my taking part in this study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

This completes Part 1 of the Information Sheet.

If the information in Part 1 has interested you and you would like to participate, please continue to read the additional information in Part 2 before making any decision.

PART 2

15. What if new information becomes available?

Sometimes during the course of a study, new information becomes available on the procedures that are being studied (such as new techniques for taking samples). If this happens, we will tell you about it and discuss with you whether you want to or should continue in the study. If you decide to withdraw, you will suffer no adverse effects as a result. If you decide to continue in the study you will be asked to sign an updated consent form.

On receiving new information, we might consider it to be in your best interests to withdraw you from the study. If so, we will explain the reasons and arrange for your care to continue. If the study is stopped for any other reason, you will be told why.

16. Will my part in this study be kept confidential?

You will be allocated a participant ID, which will be used as a code to identify you on all study forms and samples. The participant ID, address and contact email/telephone numbers will be recorded for the purpose of pollution exposure monitoring, and if appropriate, potential house visit to carry out lung function and sputum induction. Data will be stored in paper and electronic formats - all de-identified and securely stored within the research institute.

If you consent to take part in this study, the records obtained while you are in this study as well as related health records will remain strictly confidential at all times. The information will be held securely on paper and electronically at the research centre and the Royal London Hospital under the provisions of the 1998 Data Protection Act. Your name will not be passed to anyone else outside the research team or the sponsor, who is not involved in the study.

Data generated will be analysed by the research team at the Blizard institute. Data will be securely locked and only accessed by the research team. You sample records will be available to people authorised to work on the study but may also need to be made available to people authorised by the Research Sponsor, which is the organisation responsible for ensuring that the study is carried out correctly.

The information collected about you may also be shown to authorised people from the UK Regulatory Authority; this is to ensure that the study is carried out to the highest possible scientific standards. All will have a duty of confidentiality to you as a research participant.

If you withdraw consent from further study involvement, we will seek your permission to include your sample results within the study. We will not do so without your permission. Unless you object, your data and samples will remain on file and will be included in the final study analysis.

In line with Good Clinical Practice guidelines, at the end of the study, your data will be securely archived for a minimum of 20 years. Arrangements for confidential destruction will then be made.

Queen Mary University of London is the sponsor for this study based in the United Kingdom. We will be using information from you in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. Queen Mary University of London will keep identifiable information about you for 20 years.

Your rights to access change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible.

You can find out more about how we use your information at http://www.jrmo.org.uk/

Researchers from Queen Mary University of London will collect information from you for this research study in accordance with our instructions.
Queen Mary University of London will keep your name and contact details confidential and will not pass this information to Barts Health NHS Trust. Queen Mary University of London will use this information as needed, to contact you about the research study, and make sure that relevant information about the study is recorded for your care, and to oversee the quality of the study. Certain individuals from Barts Health NHS Trust and regulatory organisations may look at your medical and research records to check the accuracy of the research study. Barts Health NHS Trust will only receive information without any identifying information. The people who analyse the information will not be able to identify you and will not be able to find out your name, or contact details.
Queen Mary University of London will keep identifiable information about you from this study for 20 years after the study has finished.

17. What will happen to any samples I give?

Samples will be stored anonymously and processed as stated above, within the Blizard Institute.
The pollution monitoring badges will be analysed in Sweden (IVL Swedish Environmental Research Institute Ltd, P.O. Box 53021, SE-400 14 Gothenburg, Sweden). No samples will be sent to this location. The devices will only have anonymised data prior to transport.

Sputum and urine samples will be transported to a laboratory in Poland (Department of Medicine, Jagiellonian University Medical School, ul. św. Anny 12, 31-008 Kraków,

Poland). Samples will be anonymised using the participant ID assigned to participants prior to transport.

The samples are likely to be used up for the intended analysis, however, should there be remaining samples, 1ml aliquot of the sputum and urine may be stored anonymously in case of repeated experiments within the study period. All samples will be discarded at the end of the study (if not already discarded at the end of the intended analysis).

18. What will happen to the results of this study?

The results of the study will be available after it finishes and will usually be published in a medical journal or be presented at a scientific conference. The data will be anonymous and none of the children/ young people involved will be identified in any report or publication. Should you wish to see the results, or the publication, please ask your study doctor.

19. Who is organising and funding this study?

The study is co-sponsored by Barts and the London School of Medicine, and it is funded by Barts Charity (https://bartscharity.org.uk/).

20. Who has reviewed the study?

Before any research goes ahead it has to be checked by an independent Research Ethics Committee. They make sure that the research is fair. This project has been checked by the East Midlands Nottingham 1 Research Ethics Committee. (REC reference: **17/EM/0023**)

21. Contact for further information

You are encouraged to ask any questions you wish, before, during or after your participation. If you have any questions about the study, please speak to the research team, who will be able to provide you with up to date information about the drug(s)/procedure(s) involved. If you wish to read the research on which this study is based, please ask your research team. If you require any further information or have any concerns while taking part in the study please contact the research team (contact details are at the end of this sheet).

If you decide you would like to take part then please read and sign the consent form, which will be the only document linking your name to the participant ID. You will be given a copy of this information sheet and the consent form to keep. One copy of the consent form will be given to you, one copy to be stored in medical records and one copy to be kept within the investigator's site file located at Blizard Institute (Queen Mary University of London). The investigator site file will be securely locked and only accessed by the research doctors.

Thank you for taking the time to read this information sheet and to consider this study.

Research Team:

Chief Investigator:
Professor Jonathan Grigg, 07787 550775, j.grigg@qmul.ac.uk

Research Fellow:
Dr Norrice Liu, 020 7882 2616, n.liu@qmul.ac.uk

For advice about taking part in research in the NHS:
INVOLVE
Wessex House
Upper Market Street
Eastleigh
Hampshire
SO50 9FD
Telephone: 023 8065 1088
Textphone: 023 8062 6239
Fax: 023 8065 2885
Email: admin@invo.org.uk

For advice about research and patient issues at The Royal London Hospital and Barts Health NHS Trust:
Patient Advice and Liaison Service (PALS):
Ground Floor, Front Block
The Royal London Hospital
Whitechapel Road
London E1 1BB
Tel: 020 7943 1335
Fax: 020 7377 7361
Minicom: 020 7943 1350
E-mail: PALS@bartshealth.nhs.uk

APPENDIX 1:

Side effects of Ibuprofen:

General side-effects
Uncommon
Gastrointestinal discomfort; hypersensitivity; rash (discontinue); skin reactions
Rare or very rare
Angioedema; dyspnoea
Frequency not known
Asthma
Specific side-effects
Uncommon
Headache; nausea
Rare or very rare
acute kidney injury; agranulocytosis; anaemia; constipation; diarrhoea; fatigue; fever; gastrointestinal disorders; haemorrhage; hypotension; influenza like illness; leucopenia; liver disorder; meningitis aseptic (patients with connective-tissue disorders such as systemic lupus erythematosus may be especially susceptible); oedema; oral disorders; pancytopenia; renal papillary necrosis; respiratory disorders; severe cutaneous adverse

reactions (SCARs); shock; tachycardia; throat pain; thrombocytopenia; vomiting

Frequency not known

Crohn's disease; fertility decreased female; fluid retention; heart failure; hypertension; increased risk of arterial thromboembolism; renal failure (more common in patients with pre-existing renal impairment); respiratory tract reaction.

<CF AGE 12-15 > CHILD INFORMATION SHEET Version 1.5 30/10/2018
TO BE READ BY PARENTS AND CHILD ALONG WITH PARENT INFORMATION SHEET V 1.6
REC reference: 17/EM/0023
IRAS number: 215879
Name of researcher: Dr Norrice Liu

You are being invited to take part in a research study. Before you decide whether or not to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully, and discuss it with your parents and doctors.

Research

Research is a way we try to find the answers.

This research project is looking at what is in the air we breathe. Engines in cars and lorries burn fuels that cause AIR POLLUTION. Air pollution can get into the cells in your lungs.

Why?

We are doing a RESEARCH project to measure AIR POLLUTION in the cells in your lungs.

We want to find answers for these questions:

1. How much air pollution are you exposed to?
2. How does air pollution affect the cells in the lungs?
3. Are there certain things that will make it more difficult for your body to get rid of air pollution?

We chose you because you live in a London which is a big city with lots of cars and traffic and you have a condition called Cystic Fibrosis.

Is it ok if we....

Ask you and your parents some questions?
Test your pee?
Ask you to breathe into a tube to test your breathing?
Give you some blue puffer and.....
Ask you to breathe in salty water vapour, then cough and spit?
Ask you to take a new medicine for a few days?

Do I have to?

It's totally up to you and your parents!

What do I have to do?

Pollution Monitor

Pollution Monitoring Badge

1. We will ask you a few questions about your daily activities.
2. We will ask you to help us monitor how much Air Pollution you are exposed to- by carrying a small bag with a pollution monitor in for 2 days and 2 nights, and wear a small badge on your school bag for 2 weeks.
3. We're going to ask you to blow into a tube to test how strong your breathing is.
4. You will then breathe in some salty water vapour, then cough and spit into a pot, this should take not more than 30 minutes.
5. We will also ask you to pee into a pot.
6. We will ask you to take a new medicine for a few days, and then repeat the spitting and peeing into a pot.

Will it make me feel sick?
No – that's unusual.
Sometimes it can make you wheezy – so we will give you some blue puffer first. Blue puffer can make your heart beat faster but that's ok.

We then take your **sputum (spit)** and **urine (pee)** to our Laboratory for experiments…

What are the benefits of taking part?

We cannot promise the study will help you but the information we get might help us understand how AIR POLLUTION affects children in the future.

If at any time you don't want to do the research anymore, just tell your parents, the doctor or a nurse. Nobody will mind or be upset.

If something goes wrong and you feel sick tell a grown up straight away. People who know what to do will be there to help you.

Before any research is allowed to happen, it has to be checked by a group of people called a Research Ethics Committee. They make sure that the research is fair.

Decision time

Ok! I'm happy to take part.
Mum or Dad or a Grown-up will say yes on the grown up form… and we will be in touch to arrange the next visit…

No thank you!
We will see you next time, thanks for reading this…

Contact info
Dr Norrice Liu
Email n.liu@qmul.ac.uk
Tel: 020 7882 2616

Appendix 6 – CF study: Examples of consent and assent forms

Reducing the effects of air pollution on children with cystic fibrosis
REC reference: 17/EM/0023 IRAS number: 215879
Name of researcher: Dr Norrice Liu
CF Participants (Age 16-17)

Participant ID: _____

CONSENTFORM

	ParticipantconsentformVersion1.7 30/10/2018	**Please initial all boxes**
1	IconfirmthatIhavereadandunderstoodtheinformationsheetdated30 /10/2018 version 1.7 for the above study. I have had the opportunity to consider the information,askquestionsandhavehadtheseansweredsatisfactorily.	
2	Iunderstandthatmyparticipationisvoluntaryandthat Iamfreetowithdrawatany time,wit houtgivingareason,andwithoutmycareorlegalrightsbeingaffected.	
3	Iunderstandthatrelevantsectionsofmymedicalnotesanddatacollectedduringthe study may be looked at by research doctors from Queen Mary University London, fromregulato ryauthoritiesorfromtheNHSTrust,whereitisrelevantformytaking partinthisresearch.Igivepermissionforthesedoctorstohaveaccessmyrecords.	
4	Iagreetoanswerquestionsregardingmyexposuretoairpollution.	
5	I agree to use poll ution monitors, including a GPS device, to evaluate my personal exposure to air pollution. I understand the monitor badge will be analysed in a laboratory in Sweden but the device will only have anonymised data prior to transport.	
6	I agree to have my sp utum and urine samples collected. I understand the urine samplesandpartofthesputumsampleswillbeanonymisedandsenttoalaboratory inPolandforanalysis,andallsampleswillbedisposedofbytheendofthestudy.	
7	Iagreetotakeastandard over-the-countercourseofIbuprofen,followedbyrepeat sputumandurinecollection.	
8	Iagreeformychildtotakepartinthisstudy,he/shewillbeidentifiedbyaparticipant IDwithinthestudy.	

NameofParticipant	Signature	Date
NameofResearcher	Signature	Date

1 for participant; 1 for researcher site file; 1 (original) to be kept in medical notes.

Reducing the effects of air pollution on children with cystic fibrosis

REC reference: 17/EM/0023
IRAS number: 215879
Name of researcher: Dr Norrice Liu

CF Participants (Age 12-15)

Participant ID: _____

ASSENT FORM

	Participant assent form Version 1.5 30/10/18	Please tick ✓
1	I have read the information sheet dated 30/10/18 version 1.5 for the above study.	
2	Somebody else explained this project to me.	
3	I understand what this project is about.	
4	I have asked all the questions I want to.	
5	I understand the answers to my questions.	
6	I understand it is OK to stop taking part at any time.	
7	I am happy to take part. I am happy to give a sputum and a urine sample.	
8	I am happy to take the medicine (Ibuprofen) and give another sputum (phlegm) and urine (pee) samples.	

If you want to take part in the project, please write your name and today's date.

_____ _____ _____

Name of Child Signature Date

Thank you so much for your help!! ☺

_____ _____ _____

Name of Researcher Signature Date

Appendix 7 – Ethics approval and correspondence

East Midlands - Nottingham 1 Research Ethics Committee
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

24 January 2017

Professor Jonathan Grigg
Blizard institute
Queen Mary University of London
4 Newark Street, London
E1 2AT

Dear Professor Grigg,

Study title:	**Reducing the effects of air pollution on children with cystic fibrosis**
REC reference:	**17/EM/0023**
IRAS project ID:	**215879**

Thank you for your letter of 20th January 2017, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact hra.studyregistration@nhs.net outlining the reasons for your request.
Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise). Guidance on applying for NHS permission for research is available in the Integrated Research Application System, www.hra.nhs.uk or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from the HRA. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

The Committee has not yet completed any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. We will write to you again as soon as an SSA application(s) has been reviewed. In the meantime no study procedures should be initiated at non-NHS sites.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Contract/Study Agreement [Sponsorship agreement]	1.0	
Covering letter on headed paper [REC cover letter]	1	19 January 2017
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Sponsor Indemnity]	1.0	
IRAS Application Form [IRAS_Form_05122016]		05 December 2016
IRAS Checklist XML [Checklist_20012017]		20 January 2017
Letter from funder [Barts Charity letter]		14 March 2016
Letter from sponsor [Sponsorship letter]	1.0	02 December 2016
Other [Case Report Form]	1.0	19 September 2016
Other [Chinedu Nwokoro CV]	1.0	19 January 2016
Other [Air Pollution and CF Protocol]	1.5	19 January 2017
Other [Cover letter to REC]	1	19 January 2017
Other [Lone working guidance (NHS)]	1	19 January 2017
Other [Lone working guidance (QMUL)]	1	19 January 2017
Participant consent form [Control Consent Form Age 1-6]	1.3	30 November 2016
Participant consent form [Control Consent Form Age 7-11]	1.3	30 November 2016
Participant consent form [Control Consent Form Age 12-15]	1.3	30 November 2016
Participant consent form [CF assent 1-6]	1.1	19 January 2017
Participant consent form [CF assent 7-11]	1.5	19 January 2017
Participant consent form [CF assent 12-15]	1.4	19 January 2017
Participant consent form [Control assent 1-6]	1.2	19 January 2017
Participant consent form [Control assent 7-11]	1.5	19 January 2017
Participant consent form [Control assent 12-15]	1.4	19 January 2017
Participant consent form [CF Consent 1-6]	1.4	19 January 2017
Participant consent form [CF Consent 7-11]	1.4	19 January 2017
Participant consent form [CF Consent 12-15]	1.4	19 January 2017
Participant consent form [CF Consent 16-17]	1.5	19 January 2017
Participant consent form [Control Consent 1-6]	1.4	19 January 2017
Participant consent form [Control Consent 7-11]	1.4	19 January 2017

Participant consent form [Control Consent 12-15]	1.4	19 January 2017
Participant consent form [Control Consent 16-17]	1.5	19 January 2017
Participant information sheet (PIS) [Child PIS CF 7-11]	1.5	19 January 2017
Participant information sheet (PIS) [Child PIS CF 12-15]	1.4	19 January 2017
Participant information sheet (PIS) [Child PIS control 7-11]	1.5	19 January 2017
Participant information sheet (PIS) [Child PIS control 12-15]	1.4	19 January 2017
Participant information sheet (PIS) [PIS CF 1-6]	1.4	19 January 2017
Participant information sheet (PIS) [PIS CF 7-11]	1.4	19 January 2017
Participant information sheet (PIS) [PIS CF 12-15]	1.4	19 January 2017
Participant information sheet (PIS) [PIS CF 16-17]	1.5	19 January 2017
Participant information sheet (PIS) [PIS control 1-6]	1.4	19 January 2017
Participant information sheet (PIS) [PIS control 7-11]	1.4	19 January 2017
Participant information sheet (PIS) [PIS control 12-15]	1.4	19 January 2017
Participant information sheet (PIS) [PIS control 16-17]	1.5	19 January 2017
Sample diary card/patient card [Activity Diary]	1.0	27 September 2016
Summary CV for Chief Investigator (CI) [Jonathan Grigg CV]		
Summary CV for student [Norrice Liu CV]		

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

Notifying substantial amendments
Adding new sites and investigators
Notification of serious breaches of the protocol
Progress and safety reports
Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

17/EM/0023 **Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely,

Mr John Aldridge
Chair

Email: NRESCommittee.EastMidlands-Nottingham1@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Dr Sally Burtles*

Elizabeth Clough

NHS
Health Research Authority

Professor Jonathan Grigg Blizard Institute Email: hra.approval@nhs.net
Queen Mary University of London
4 Newark Street, London
E1 2AT

15 February 2017
Amended and Reissued 22 February 2017

Dear Professor Grigg,

Letter of HRA Approval

Study title:	**Reducing the effects of air pollution on children with cystic fibrosis**
IRAS project ID:	**215879**
REC reference:	**17/EM/0023**
Sponsor	**Queen Mary University of London**

I am pleased to confirm that **HRA Approval** has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications noted in this letter.

Participation of NHS Organisations in England
The sponsor should now provide a copy of this letter to all participating NHS organisations in England.

Appendix B provides important information for sponsors and participating NHS organisations in England for arranging and confirming capacity and capability. **Please read *Appendix B* carefully**, in particular the following sections:
Participating NHS organisations in England – this clarifies the types of participating organisations in the study and whether or not all organisations will be undertaking the same activities
Confirmation of capacity and capability - this confirms whether or not each type of participating NHS organisation in England is expected to give formal confirmation of capacity and capability. Where formal confirmation is not expected, the section also provides details on the time limit given to participating organisations to opt out of the study, or request additional time, before their participation is assumed.
Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria) - this provides detail on the form of agreement to be used in the study to confirm capacity and capability, where applicable.
Further information on funding, HR processes, and compliance with HRA criteria and standards is also provided.

It is critical that you involve both the research management function (e.g. R&D office) supporting each organisation and the local research team (where there is one) in setting up your study. Contact details and further information about working with the research

management function for each organisation can be accessed from www.hra.nhs.uk/hra-approval.

Appendices
The HRA Approval letter contains the following appendices:
A – List of documents reviewed during HRA assessment
B – Summary of HRA assessment

After HRA Approval
The document *"After Ethical Review – guidance for sponsors and investigators",* issued with your REC favourable opinion, gives detailed guidance on reporting expectations for studies, including:
Registration of research
Notifying amendments
Notifying the end of the study
The HRA website also provides guidance on these topics, and is updated in the light of changes in reporting expectations or procedures.

In addition to the guidance in the above, please note the following:
HRA Approval applies for the duration of your REC favourable opinion, unless otherwise notified in writing by the HRA.
Substantial amendments should be submitted directly to the Research Ethics Committee, as detailed in the *After Ethical Review* document. Non-substantial amendments should be submitted for review by the HRA using the form provided on the HRA website, and emailed to hra.amendments@nhs.net.
The HRA will categorise amendments (substantial and non-substantial) and issue confirmation of continued HRA Approval. Further details can be found on the HRA website.

Scope
HRA Approval provides an approval for research involving patients or staff in NHS organisations in England.

If your study involves NHS organisations in other countries in the UK, please contact the relevant national coordinating functions for support and advice. Further information can be found at http://www.hra.nhs.uk/resources/applying-for-reviews/nhs-hsc-rd-review/.

If there are participating non-NHS organisations, local agreement should be obtained in accordance with the procedures of the local participating non-NHS organisation.

User Feedback
The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please email the HRA at hra.approval@nhs.net. Additionally, one of our staff would be happy to call and discuss your experience of HRA Approval.

HRA Training
We are pleased to welcome researchers and research management staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

Your IRAS project ID is **215879**. Please quote this on all correspondence.

Yours sincerely

Thomas Fairman
HRA Assessor

Email: hra.approval@nhs.net

Copy to: Dr Sally Burtles, Barts Health NHS Trust, (Sponsor Contact)
Ms Elizabeth Clough, Barts Health NHS Trust, (lead NHS R&D Contact)

Appendix A - List of Documents

The final document set assessed and approved by HRA Approval is listed below.

Document	*Version*	*Date*
Contract/Study Agreement [Sponsorship agreement]	1.0	12 October 2016
Covering letter on headed paper [REC cover letter]		19 January 2017
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Sponsor Indemnity]		15 July 2016
IRAS Application Form [IRAS_Form_05122016]		05 December 2016
Letter from funder [Barts Charity letter]		14 March 2016
Letter from sponsor [Sponsorship letter]	2.0	02 December 2016
Other [Case Report Form]	1.0	19 September 2016
Other [Chinedu Nwokoro CV]	1.0	19 January 2016
Other [Air Pollution and CF Protocol]	1.5	19 January 2017
Other [Lone working guidance (NHS)]	2	01 January 2009
Other [Lone working guidance (QMUL)]		01 September 2014
Other [Confirmation from site re Statement of Activities and Schedule of Events]		14 February 2017
Other [Sponsor confirmation of non-substantial amendment]		15 February 2017
Participant consent form [Assent Form CF 1 - 6]	1.1	30 November 2016
Participant consent form [Assent Form CF 7 - 11]	1.5	19 January 2017
Participant consent form [Assent Form CF 12 - 15]	1.4	19 January 2017
Participant consent form [Assent Form Control 1 - 6]	1.2	19 January 2017
Participant consent form [Assent Form Control 7 - 11]	1.5	19 January 2017
Participant consent form [Assent Form Control 12 - 15]	1.4	19 January 2017
Participant consent form [Consent Form CF 1 - 6]	1.5	14 February 2017
Participant consent form [Consent Form CF 7 - 11]	1.5	14 February 2017
Participant consent form [Consent Form CF 12 - 15]	1.5	14 February 2017
Participant consent form [Consent Form CF 16 - 17]	1.6	14 February 2017

Participant consent form [Consent Form Control 1 - 6]	1.5	14 February 2017
Participant consent form [Consent Form Control 7 - 11]	1.5	14 February 2017
Participant consent form [Consent Form Control 12 - 15]	1.5	14 February 2017
Participant consent form [Consent Form Control 16 - 17]	1.6	14 February 2017
Participant information sheet (PIS) [Child PIS CF 1 - 6]	1.2	20 February 2017
Participant information sheet (PIS) [Child PIS CF 7 - 11]	1.5	19 January 2017
Participant information sheet (PIS) [Child PIS CF 12 - 15]	1.4	19 January 2017
Participant information sheet (PIS) [Child PIS Control 1 - 6]	1.2	20 February 2017
Participant information sheet (PIS) [Child PIS Control 7 - 11]	1.5	19 January 2017
Participant information sheet (PIS) [Child PIS Control 12 - 15]	1.4	19 January 2017
Participant information sheet (PIS) [Parents PIS CF 1 - 6]	1.5	14 February 2017
Participant information sheet (PIS) [Parents PIS CF 7 - 11]	1.5	14 February 2017
Participant information sheet (PIS) [Parents PIS CF 12 - 15]	1.5	14 February 2017
Participant information sheet (PIS) [PIS CF 16 - 17]	1.6	14 February 2017
Participant information sheet (PIS) [Parents PIS Control 1 - 6]	1.5	14 February 2017
Participant information sheet (PIS) [Parents PIS Control 7 - 11]	1.5	14 February 2017
Participant information sheet (PIS) [Parents PIS Control 12 - 15]	1.5	14 February 2017
Participant information sheet (PIS) [PIS Control 16 - 17]	1.6	14 February 2017
Sample diary card/patient card [Activity Diary]	1.0	27 September 2016
Summary CV for Chief Investigator (CI) [Jonathan Grigg CV]		26 October 2016
Summary CV for student [Norrice Liu CV]		26 October 2016

Appendix B - Summary of HRA Assessment

This appendix provides assurance to you, the sponsor and the NHS in England that the study, as reviewed for HRA Approval, is compliant with relevant standards. It also provides information and clarification, where appropriate, to participating NHS organisations in England to assist in assessing and arranging capacity and capability.
For information on how the sponsor should be working with participating NHS organisations in
England, please refer to the, *participating NHS organisations, capacity and capability* and *Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria)* sections in this appendix.
The following person is the sponsor contact for the purpose of addressing participating organisation questions relating to the study:

Name: Dr Sally Burtles
Email: sponsorsrep@bartshealth.nhs.uk

HRA assessment criteria

Section	HRA Assessment Criteria	Compliant with Standards	Comments
1.1	IRAS application completed correctly	Yes	No comments
2.1	Participant information/consent documents and consent process	Yes	No comments
3.1	Protocol assessment	Yes	No comments
4.1	Allocation of responsibilities and rights are agreed and documented	Yes	This is a non-commercial single site study taking place in the NHS where that single NHS organisation has confirmed that no study agreements are required. Therefore no Statement of Activities and Schedule of Events has been submitted.
4.2	Insurance/indemnity arrangements assessed	Yes	Where applicable, independent contractors (e.g. General Practitioners) should ensure that the professional indemnity provided by their medical defence organisation covers the activities expected of them for this
Section	**HRA Assessment Criteria**	**Compliant with Standards**	**Comments**

			research study
4.3	Financial arrangements assessed	Yes	External study funding has been secured from Barts Charity.
5.1	Compliance with the Data Protection Act and data security issues assessed	Yes	No comments
5.2	CTIMPS – Arrangements for compliance with the Clinical Trials Regulations assessed	Not Applicable	No comments
5.3	Compliance with any applicable laws or regulations	Yes	No comments
6.1	NHS Research Ethics Committee favourable opinion received for applicable studies	Yes	REC Favourable Opinion was issued by the Nottingham 1 Research Ethics Committee on the 24th January 2017 Amended documents were submitted on by the researchers to comply with HRA Approval standards. These were classified by the sponsor as a nonsubstantial amendment.
6.2	CTIMPS – Clinical Trials Authorisation (CTA) letter received	Not Applicable	No comments
6.3	Devices – MHRA notice of no objection received	Not Applicable	No comments
6.4	Other regulatory approvals and authorisations received	Not Applicable	No comments

Participating NHS Organisations in England

This provides detail on the types of participating NHS organisations in the study and a statement as to whether the activities at all organisations are the same or different.
This is a non-commercial single site study taking place in the NHS. Therefore there is only one site type involved in the research.

If this study is subsequently extended to other NHS organisation(s) in England, an amendment should be submitted to the HRA, with a Statement of Activities and Schedule of Events for the newly participating NHS organisation(s) in England.

The Chief Investigator or sponsor should share relevant study documents with participating NHS organisations in England in order to put arrangements in place to deliver the study.

The documents should be sent to both the local study team, where applicable, and the office providing the research management function at the participating organisation. For NIHR CRN Portfolio studies, the Local LCRN contact should also be copied into this correspondence. For further guidance on working with participating NHS organisations please see the HRA website.

If chief investigators, sponsors or principal investigators are asked to complete site level forms for participating NHS organisations in England which are not provided in IRAS or on the HRA website, the chief investigator, sponsor or principal investigator should notify the HRA immediately at hra.approval@nhs.net. The HRA will work with these organisations to achieve a consistent approach to information provision.

Confirmation of Capacity and Capability

This describes whether formal confirmation of capacity and capability is expected from participating NHS organisations in England.
NHS organisations in England that are participating in the study **will be expected to formally confirm their capacity and capability** to host this research. Following issue of this letter, participating NHS organisations in England may now confirm to the sponsor their capacity and capability to host this research, when ready to do so. How capacity and capacity will be confirmed is detailed in the Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria) section of this appendix. The Assessing, Arranging, and Confirming document on the HRA website provides further information for the sponsor and NHS organisations on assessing, arranging and confirming capacity and capability.

Principal Investigator Suitability

This confirms whether the sponsor position on whether a PI, LC or neither should be in place is correct for each type of participating NHS organisation in England and the minimum expectations for education, training and experience that PIs should meet (where applicable).
A Principal Investigator should be appointed at study sites GCP training is not a generic training expectation, in line with the HRA statement on training expectations.

HR Good Practice Resource Pack Expectations

This confirms the HR Good Practice Resource Pack expectations for the study and the pre-engagement checks that should and should not be undertaken
If members of the external research team will be attending NHS sites to conduct the study activities

detailed at IRAS A18 and A19 they should obtain an Honorary Research Contract for this purpose from one NHS organisation, followed by Letters of Access for subsequent organisations. This would be on the basis of a Research Passport or an NHS to NHS confirmation of pre-engagement checks letter (if NHS employed). Pre-engagement checks should confirm standard/enhanced DBS checks, appropriate barred list checks, and occupational health clearance.

For research team members only administering questionnaires or surveys, a Letter of Access based on enhanced DBS checks, appropriate barred list checks and occupational health clearance would be appropriate.

Other Information to Aid Study Set-up

This details any other information that may be helpful to sponsors and participating NHS organisations in England to aid study set-up.
The applicant has indicated that they do not intend to apply for inclusion on the NIHR CRN Portfolio.

NHS
Health Research Authority

East Midlands - Nottingham 1 Research Ethics Committee
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Please note: This is the favourable opinion of the REC only and does not allow the amendment to be implemented at NHS sites in England until the outcome of the HRA assessment has been confirmed.

19 October 2017

Professor Jonathan Grigg
Blizard institute
Queen Mary University of London
4 Newark street, London
E1 2AT

Dear Professor Grigg

Study title:	**Reducing the effects of air pollution on children with cystic fibrosis**
REC reference:	**17/EM/0023**
Amendment number:	**1**
Amendment date:	**28 September 2017**
IRAS project ID:	**215879**

The above amendment was reviewed at the meeting of the Sub-Committee held in correspondence on 16 October 2017.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Covering letter on headed paper [REC cover letter - amendment.docx]		28 September 2017
Notice of Substantial Amendment (non-CTIMP) [CF amendment IRAS form final.pdf]	1	28 September 2017

Other [Conversation with applicant over re-consenting.pdf]		05 October 2017
Research protocol or project proposal [Air Pollution and CF Protocol 1.6 clean.docx]	1.6	26 October 2017
Research protocol or project proposal [Air Pollution and CF Protocol 1.6 tracking.docx]	1.6	26 October 2017

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

Working with NHS Care Organisations

Sponsors should ensure that they notify the R&D office for the relevant NHS care organisation of this amendment in line with the terms detailed in the categorisation email issued by the lead nation for the study.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our Research Ethics Committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

17/EM/0023:	**Please quote this number on all correspondence**

Yours sincerely

Professor Cris Constantinescu Chair

E-mail: NRESCommittee.EastMidlands-Nottingham1@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Elizabeth Clough, Barts Health NHS Trust Dr Sally Burtles*

East Midlands - Nottingham 1 Research Ethics Committee

Attendance at Sub-Committee of the REC meeting on 16 October 2017

Committee Members:

Name	*Profession*	*Present*
Professor Cris Constantinescu (Chair)	Professor of Clinical Neurology	Yes
Ms Stephanie Sampson	PhD Student	Yes

Also in attendance:

Name	*Position (or reason for attending)*
Mr George R. Martin	REC Assistant (Minutes)

NHS
Health Research Authority

East Midlands - Nottingham 1 Research Ethics Committee

The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Please note: This is the favourable opinion of the REC only and does not allow the amendment to be implemented at NHS sites in England until the outcome of the HRA assessment has been confirmed.

27 April 2018

Dr Norrice Liu
Clinical Research Fellow
Blizard Institute
4 Newark Street
Whitechapel
E1 2AT

Dear Dr Liu

Study title:	**Reducing the effects of air pollution on children with cystic fibrosis**
REC reference:	**17/EM/0023**
Amendment number:	**2**
Amendment date:	**04 April 2018**
IRAS project ID:	**215879**

The above amendment was reviewed on 24 April 2018 by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Decision: No ethical issues.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Covering letter on headed paper [REC cover letter - amendment 2 04132018.docx]	N/a	04 April 2018
Notice of Substantial Amendment (non-CTIMP) [AmendmentForm_ReadyForSubmission 20180416.pdf]	2	04 April 2018
Other [Bronchiectasis assent form (7-11).docx]	1.0	04 April 2018
Other [Bronchiectasis assent form (12-15).docx]	1.0	04 April 2018
Participant consent form [Bronchiectasis consent form (7-11).docx]	1.0	04 April 2018
Participant consent form [Bronchiectasis consent form (12-15).docx]	1.0	04 April 2018
Participant consent form [Bronchiectasis consent form (16-17).docx]	1.0	04 April 2018
Participant information sheet (PIS) [Bronchiectasis child info sheet (7-11) clean.docx]	1.0	04 April 2018
Participant information sheet (PIS) [Bronchiectasis child infor sheet (12-15) clean.docx]	1.0	04 April 2018
Participant information sheet (PIS) [Bronchiectasis info sheet (1215) clean.docx]	1.0	04 April 2018
Participant information sheet (PIS) [Bronchiectasis infor sheet (1617) clean.docx]	1.0	04 April 2018
Participant information sheet (PIS) [Bronchiectasis info sheet (7-11) clean.docx]	1.0	04 April 2018
Research protocol or project proposal [Air Pollution and CF Protocol 1.7 clean.docx]	1.7	04 April 2018
Research protocol or project proposal [Air Pollution and CF Protocol 1.7 tracking.docx]	1.7	04 April 2018

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

Working with NHS Care Organisations

Sponsors should ensure that they notify the R&D office for the relevant NHS care organisation of this amendment in line with the terms detailed in the categorisation email issued by the lead nation for the study.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our Research Ethics Committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

17/EM/0023:	**Please quote this number on all correspondence**

Yours sincerely

Pp

Ellen Milazzo Chair

E-mail: NRESCommittee.EastMidlands-Nottingham1@nhs.net

Enclosures: List of names and professions of members who took part in the review

Copy to: Elizabeth Clough, Barts Health NHS Trust
Dr Norrice Liu, Blizard Institute
East Midlands - Nottingham 1 Research Ethics Committee

Attendance at Sub-Committee of the REC meeting on 24 April 2018

Committee Members:

Name	*Profession*	*Present*
Dr Sarah Forster	Respiratory Registrar	Yes
Ms Ellen Milazzo (Chair)	Development and Change Management Consultant	Yes

Also in attendance:

Name	*Position (or reason for attending)*
Ellena Stansbury	REC Assistant (Minutes)

East Midlands - Nottingham 1 Research Ethics Committee
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Please note: This is the favourable opinion of the REC only and does not allow the amendment to be implemented at NHS sites in England until the outcome of the HRA assessment has been confirmed.

17 September 2018

Dr Norrice Liu
Clinical Research Fellow
Blizard Institute
4 Newark Street
Whitechapel
E1 2AT

Document	*Version*	*Date*
Covering letter on headed paper [REC cover letter - amendment	3	23 August 2018

Dear Dr Liu

Study title:	**Reducing the effects of air pollution on children with cystic fibrosis**
REC reference:	**17/EM/0023**
Amendment number:	**3**
Amendment date:	**23 August 2018**
IRAS project ID:	**215879**

The above amendment was reviewed on 11 September 2018 by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Decision: No ethical issues.

Approved documents

The documents reviewed and approved at the meeting were:

3.docx]		
Notice of Substantial Amendment (non-CTIMP) [AmendmentForm_snapshot20180823.pdf]	3	23 August 2018
Research protocol or project proposal [Air Pollution and CF Protocol 1.8 tracking.docx]	1.8	25 June 2018
Research protocol or project proposal [Air Pollution and CF Protocol 1.8 clean.docx]	1.8	25 June 2018

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

Working with NHS Care Organisations

Sponsors should ensure that they notify the R&D office for the relevant NHS care organisation of this amendment in line with the terms detailed in the categorisation email issued by the lead nation for the study.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our Research Ethics Committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

17/EM/0023:	**Please quote this number on all correspondence**

Yours sincerely

Pp
Mr Murthy Nyasavajjala Chair

E-mail: NRESCommittee.EastMidlands-Nottingham1@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Elizabeth Clough, Barts Health NHS Trust Dr Norrice Liu, Blizard Institute*
East Midlands - Nottingham 1 Research Ethics Committee

Attendance at Sub-Committee of the REC meeting on 11 September 2018

Committee Members:

Name	*Profession*	*Present*	*Notes*
Dr Blerina Kellezi	Lecturer in Psychology	Yes	
Mr Murthy Nyasavajjala	Consultant Upper GI and General Surgery	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Ellena Stansbury	REC Assistant

NHS
Health Research Authority

East Midlands - Nottingham 1 Research Ethics Committee
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Please note: This is the favourable opinion of the REC only and does not allow the amendment to be implemented at NHS sites in England until the outcome of the HRA assessment has been confirmed.

08 January 2019

Dr Norrice Liu
Clinical Research Fellow
Blizard Institute
4 Newark Street
Whitechapel
E1 2AT

Document	*Version*	*Date*
Covering letter on headed paper [REC cover letter - amendment 4 .docx]	4	15 November 2018

Dear Dr Liu,

Study title:	**Reducing the effects of air pollution on children with cystic fibrosis**
REC reference:	**17/EM/0023**
Amendment number:	**4**
Amendment date:	**17 December 2018**
IRAS project ID:	**215879**

The above amendment was reviewed on 03 January 2019 by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Decision: No ethical issues.

Approved documents

The documents reviewed and approved at the meeting were:

Notice of Substantial Amendment (non-CTIMP) [AmendmentForm_ReadyForSubmission20181217.pdf]	4	17 December 2018
Participant consent form [Bronchiectasis assent form (12-15) v1.1 clean.docx]	1.1	30 October 2018
Participant consent form [Bronchiectasis assent form (12-15) v1.1 tracking.docx]	1.1	30 October 2018
Participant consent form [Bronchiectasis assent form (7-11) v1.1 clean.docx]	1.1	30 October 2018
Participant consent form [Bronchiectasis assent form (7-11) v1.1 tracking.docx]	1.1	30 October 2018
Participant consent form [Bronchiectasis consent form (12-15) v1.1 tracking.docx]	1.1	30 October 2018
Participant consent form [Bronchiectasis consent form (16-17) v1.1 clean.docx]	1.1	30 October 2018
Participant consent form [Bronchiectasis consent form (16-17) v1.1 tracking.docx]	1.1	30 October 2018
Participant consent form [Bronchiectasis consent form (7-11) v1.1 clean.docx]	1.1	30 October 2018
Participant consent form [Bronchiectasis consent form (7-11) v1.1 tracking.docx]	1.1	30 October 2018
Participant consent form [Bronchiectasis consent form (12-15) v1.1 clean.docx]	1.1	30 October 2018
Participant consent form [CF assent form (12-15) v1.5 clean REC.docx]	1.5	30 October 2018
Participant consent form [CF assent form (12-15) v1.5 tracking REC.docx]	1.5	30 October 2018
Participant consent form [CF assent form (7-11) v1.6 clean REC.docx]	1.6	30 October 2018
Participant consent form [CF assent form (7-11) v1.6 tracking REC.docx]	1.6	30 October 2018
Participant consent form [CF Consent Form (12-15) REC v1.6 clean.docx]	1.6	30 October 2018
Participant consent form [CF Consent Form (12-15) REC v1.6 tracking.docx]	1.6	30 October 2018
Participant consent form [CF Consent Form (16-17) REC v1.7 clean.docx]	1.7	30 October 2018
Participant consent form [CF Consent Form (16-17) REC v1.7 tracking.docx]	1.7	30 October 2018
Participant consent form [CF study info sheet (7-11) v1.6 cleanHRA.docx]	1.6	30 October 2018
Participant consent form [CF study info sheet (7-11) v1.6 tracking HRA.docx]	1.6	30 October 2018
Participant consent form [CF Consent Form (7-11) REC v1.6 clean.docx]	1.6	30 October 2018
Participant consent form [CF Consent Form (7-11) REC v1.6 tracking.docx]	1.6	30 October 2018
Participant consent form [Control assent form (7-11) v1.6 cleanREC.docx]	1.6	30 October 2018
Participant consent form [Control Consent Form (7-11) REC v1.6 clean.docx]	1.6	30 October 2018
Participant consent form [Control Consent Form (7-11) REC v1.6 tracking.docx]	1.6	30 October 2018

Participant consent form [Control Consent Form (12-15) REC v1.6 clean.docx]	1.6	30 October 2018
Participant consent form [Control assent form (7-11) v1.6 trackingREC.docx]	1.6	30 October 2018
Participant consent form [Control assent form (12-15) v1.5 clean REC.docx]	1.5	30 October 2018

Participant consent form [Control assent form (12-15) v1.5 tracking REC.docx]	1.5	30 October 2018
Participant consent form [Control Consent Form (16-17) REC v1.7 clean.docx]	1.7	30 October 2018
Participant consent form [Control Consent Form (16-17) REC v1.7 tracking.docx]	1.7	30 October 2018
Participant consent form [Control Consent Form (12-15) REC v1.6 tracking.docx]	1.6	30 October 2018
Participant information sheet (PIS) [Bronchiectasis child info sheet (7-11) v1.1 clean.docx]	1.1	30 October 2018
Participant information sheet (PIS) [Bronchiectasis child info sheet (7-11) v1.1 tracking.docx]	1.1	30 October 2018
Participant information sheet (PIS) [Bronchiectasis child infor sheet (12-15) v1.1 clean.docx]	1.1	30 October 2018
Participant information sheet (PIS) [Bronchiectasis info sheet (7-11) v1.1 clean.docx]	1.1	30 October 2018
Participant information sheet (PIS) [Bronchiectasis info sheet (7-11) v1.1 tracking.docx]	1.1	30 October 2018
Participant information sheet (PIS) [Bronchiectasis info sheet (1215) v1.1 clean.docx]	1.1	30 October 2018
Participant information sheet (PIS) [Bronchiectasis child infor sheet (12-15) v1.1 tracking.docx]	1.1	30 October 2018
Participant information sheet (PIS) [CF child info sheet (7-11) REC v1.1 tracking.docx]	1.1	30 October 2018
Participant information sheet (PIS) [CF child info sheet (7-11) REC v1.1 tracking.docx]	1.1	30 October 2018
Participant information sheet (PIS) [CF child info sheet (12-15) REC v1.1 clean.docx]	1.1	30 October 2018
Participant information sheet (PIS) [Bronchiectasis info sheet (1215) v1.1 tracking.docx]	1.1	30 October 2018
Participant information sheet (PIS) [Bronchiectasis infor sheet (1617) v1.1 clean.docx]	1.1	30 October 2018
Participant information sheet (PIS) [Bronchiectasis infor sheet (1617) v1.1 tracking.docx]	1.1	30 October 2018
Participant information sheet (PIS) [CF study info sheet (12-15) v1.6 clean HRA.docx]	1.6	30 October 2018
Participant information sheet (PIS) [CF child info sheet (12-15) REC v1.1 tracking.docx]	1.1	30 October 2018
Participant information sheet (PIS) [CF study info sheet (control 711) v1.6 trackingHRA.docx]	1.6	30 October 2018
Participant information sheet (PIS) [CF study info sheet (control 1215) v1.6 clean HRA.docx]	1.6	30 October 2018
Participant information sheet (PIS) [CF study info sheet (control 1215) v1.6 tracking HRA.docx]	1.6	30 October 2018
Participant information sheet (PIS) [CF study info sheet (control 1617) v1.7 cleanHRA.docx]	1.7	30 October 2018
Participant information sheet (PIS) [CF study info sheet (control 1617) v1.7 trackingHRA.docx]	1.7	30 October 2018

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Participant information sheet (PIS) [Control child infor sheet (12-15) REC v1.1 tracking.docx]	1.1	30 October 2018
Participant information sheet (PIS) [Control child infor sheet (7-11) REC v1.1 clean.docx]	1.1	30 October 2018
Participant information sheet (PIS) [Control child infor sheet (7-11) REC v1.1 tracking.docx]	1.1	30 October 2018
Research protocol or project proposal [Air Pollution and CF Protocol 1.9 clean.docx]	1.9	30 October 2018
Research protocol or project proposal [Air Pollution and CF Protocol 1.9 tracking .docx]	1.9	30 October 2018

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

Working with NHS Care Organisations

Sponsors should ensure that they notify the R&D office for the relevant NHS care organisation of this amendment in line with the terms detailed in the categorisation email issued by the lead nation for the study.

Statement of compliance

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We are pleased to welcome researchers and R & D staff at our Research Ethics Committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

17/EM/0023:	**Please quote this number on all correspondence**

Yours sincerely

Pp
Professor Cris Constantinescu Chair

E-mail: NRESCommittee.EastMidlands-Nottingham1@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Elizabeth Clough, Barts Health NHS Trust Dr Norrice Liu, Blizard Institute*

East Midlands - Nottingham 1 Research Ethics Committee

Attendance at Sub-Committee of the REC meeting on 03 January 2019

Committee Members:

Name	*Profession*	*Present*
Professor Cris Constantinescu (Chair)	Professor of Clinical Neurology	Yes
Mr Murthy Nyasavajjala	Consultant Upper GI and General Surgery	Yes

Also in attendance:

Name	*Position (or reason for attending)*
Ellena Stansbury	REC Assistant (Minutes)

NHS
Health Research Authority

East Midlands - Nottingham 1 Research Ethics Committee
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Please note: This is the favourable opinion of the REC only and does not allow the amendment to be implemented at NHS sites in England until the outcome of the HRA assessment has been confirmed.

28 February 2019

Dr Norrice Liu
Clinical Research Fellow
Blizard Institute
4 Newark Street
Whitechapel
E1 2AT

Dear Dr Liu

Study title:	**Reducing the effects of air pollution on children with cystic fibrosis**
REC reference:	**17/EM/0023**
Amendment number:	**5**
Amendment date:	**04 February 2019**
IRAS project ID:	**215879**

The above amendment was reviewed on 26 February 2019 by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

The Sub-Committee agreed that the substantial amendment did not raise any material ethical issues. However, did ask for some clarity to be provided via email to the below queries.

Will the researcher actively reach out to this healthy cohort via the various organisations mentioned and if so, how will this be achieved?

Equally, does the team hope to recruit via self-referral method or a mixture of the two?

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Covering letter on headed paper [REC cover letter - amendment 5 .docx]	5	04 February 2019
Notice of Substantial Amendment (non-CTIMP) [AmendmentForm_ReadyForSubmission20190208.pdf]	5	04 February 2019
Research protocol or project proposal [Air Pollution and CF Protocol 1.10 tracking.docx]	1.10	04 February 2019
Research protocol or project proposal [Air Pollution and CF Protocol 1.10 clean.docx]	1.10	04 February 2019

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

Working with NHS Care Organisations

Sponsors should ensure that they notify the R&D office for the relevant NHS care organisation of this amendment in line with the terms detailed in the categorisation email issued by the lead nation for the study.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our Research Ethics Committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

17/EM/0023:	**Please quote this number on all correspondence**

Yours sincerely

Pp
Professor Cris Constantinescu Chair

E-mail: NRESCommittee.EastMidlands-Nottingham1@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Elizabeth Clough, Barts Health NHS Trust Dr Norrice Liu, Blizard Institute*

East Midlands - Nottingham 1 Research Ethics Committee

Attendance at Sub-Committee of the REC meeting on 26 February 2019

Committee Members:

Name	*Profession*	*Present*
Professor Cris Constantinescu (Chair)	Professor of Clinical Neurology	Yes
Mrs Norma Thompson	Clinical Research Nurse	Yes

Also in attendance:

Name	*Position (or reason for attending)*
Ellena Stansbury	REC Assistant (Minutes)

Appendix 8 – Protocol: Air pollution particles in placenta (APPIP)

1. GENERAL INFORMATION

Full Title	Presence of Air Pollution Particles in Placental Macrophages
Short Title/Acronym	Air Pollution Particles in Placenta (APPIP)
Sponsor:	Queen Mary, University of London Dr Mays Jawad, R&D operations manager Joint Research Management Office Queen Mary Innovation Centre 5 Walden Street London, E1 2EF Tel: 020 7882 7275 Email: sponsorsrep@bartshealth.nhs.uk
REC reference:	17/NW/0092
IRAS project ID:	219053
Chief Investigator:	Professor Jonathan Grigg Centre for Paediatrics, Blizard Institute 4 Newark Street, London E1 2AT, UK Tel: 02078822206 Fax: 02078825555 Email: j.grigg@qmul.ac.uk
Principal Investigator:	Dr Norrice Liu Centre for Paediatrics, Blizard Institute 4 Newark Street, London E1 2AT, UK Tel: 02078822616 Fax: 02078825555 Email: n.liu@qmul.ac.uk
Co-Investigator:	Professor Shakila Thangaratinam Women's Health Research Unit Multidisciplinary Evidence Synthesis Hub (MESH) The Blizard Institute 4 Newark Street, London E1 2AT, UK Mobile: 07887775891 Email: s.thangaratinam@qmul.ac.uk
Sites/ Organisations:	Barts and The London School of Medicine and Dentistry Centre for Paediatrics Blizard Institute 4 Newark Street London E1 2AT, United Kingdom Barts Health NHS Trust, Royal London Hospital Whitechapel Road London E1 1BB, United Kingdom

Laboratory:	Blizard 4 Newark Street London E1 2AT United Kingdom Tel: 020 7882 2483	Institute
Funder:	Barts Charity Ground Floor 12 Cock Lane London, EC1A 9BU Tel: 02076181717 Fax: 02072489395 Emails: blcharity@bartshealth.nhs.uk Grant Reference: MGU0312	

Contents Page

2. GLOSSARY of Terms and Abbreviations

AE	Adverse Event
AM	Airway macrophage
AR	Adverse Reaction
ASR	Annual Safety Report
BC	Black Carbon
CA	Competent Authority
CI	Chief Investigator
CRF	Case Report Form
CRO	Contract Research Organisation
DMC	Data Monitoring Committee
EC	European Commission
GAfREC	Governance Arrangements for NHS Research Ethics Committees
ICF	Informed Consent Form
IUGR	Intra-uterine growth restriction
JRMO	Joint Research Management Office
NHS REC	National Health Service Research Ethics Committee
NHS R&D	National Health Service Research & Development
Participant	An individual who takes part in a clinical trial
PI	Principal Investigator
PIS	Participant Information Sheet
PM	Particulate Matter
QA	Quality Assurance
QC	Quality Control
RCT	Randomised Controlled Trial
REC	Research Ethics Committee
SAE	Serious Adverse Event
SDV	Source Document Verification
SOP	Standard Operating Procedure
SSA	Site Specific Assessment
TMG	Trial Management Group
TSC	Trial Steering Committee

3. SIGNATURE PAGE

Chief Investigator Agreement

The clinical study as detailed within this research protocol **(Version 1.5, dated 24/04/2019)**, or any subsequent amendments will be conducted in accordance with the Research Governance Framework for Health & Social Care (2005), the World Medical Association Declaration of Helsinki (1996) and the current applicable regulatory requirements and any subsequent amendments of the appropriate regulations.

Chief Investigator Name: Professor Jonathan Grigg

Chief Investigator Site: Blizard Institute, Queen Mary University of London

Signature and Date: **24/04/2019**

Principal Investigator Agreement *(if different from Chief investigator)*

The clinical study as detailed within this research protocol **(Version 1.5, dated 24/04/2019)**, or any subsequent amendments will be conducted in accordance with the Research Governance Framework for Health & Social Care (2005), the World Medical Association Declaration of Helsinki (1996) and the current applicable regulatory requirements and any subsequent amendments of the appropriate regulations.

Principal Investigator Name: Dr Norrice Liu

Principal Investigator Site: Blizard Institute, Queen Mary University of London

Signature and Date: **24/04/2019**

4. SUMMARY/SYNOPSIS

Short Title	Air Pollution Particles in Placenta (APPIP)
Methodology	Pilot cross sectional study
Research Sites	• Barts Health NHS Trust • Blizard Institute, Queen Mary University of London
Objectives/Aims	To establish whether maternal exposure to black carbon will result in its presence in placental cells
Number of Participants/Patients	20 expectant mothers will be recruited for placental retrieval at delivery
Main Inclusion Criteria	1. Uncomplicated term pregnancy (>37 week gestation) 2. Delivery by elective Caesarean section 3. Age 18-50
Statistical Methodology and Analysis (if applicable)	This is a pilot study. 20 participants will be a feasible number regarding time and cost.
Proposed Start Date	21/11/2016
Proposed End Date	20/11/2020
Study Duration	4 years

INTRODUCTION

Background

Microscopic particles of soot (particulate matter, PM) from petrol and diesel engine emissions are linked to a wide range of adverse health effects.

Antenatal health of the baby is closely related to maternal health. Exposure to air pollution during pregnancy can affect foetal and postnatal development. Foetal cells are fast replicating and are therefore sensitive to external factors. Airway development begins at 4 weeks of gestation with alveolar development starting at around 36 weeks to early adulthood. Exposure to air pollution can disturb alveolarisation, leading to impaired lung development and function (1,2). Previous studies have reported a link between maternal pollution exposure and adverse birth outcomes such as increased infant mortality, lower birth weight, impaired lung function and later respiratory problems (3,4). Possible mechanisms include particle translocation across tissue barriers or particle penetration across cellular membranes (3). Potential trans-placental transfer of nanomaterials up to 240nm was shown to be possible (5).

Removal of PM involves phagocytosis by macrophages, this usually takes place in the airway following PM inhalation. One established way of assessing air pollution exposure is quantifying the amount of black carbon (BC) in airway macrophages (AM).

Similar idea can be applied to the defense mechanism within the placenta. Many macrophages are present in the placenta, they can be detected early in the first trimester. Placental macrophages assist in host defense as well as placental development and maintenance of pregnancy (6). Any presence of black carbon should theoretically be phagocytosed by placental macrophages.

Rationale

To date, presence of PM in the placenta has not been studied. This pilot study will look into whether maternal exposure to PM, particularly black carbon, will result in trans-placental transfer, thereby having possible direct casual effect on birth outcomes.

This study is part of a PhD project of the Principle Investigator.

STUDY OBJECTIVES

Primary Objective

I. To establish whether maternal exposure to black carbon will result in its presence in placental cells.
II. To establish whether there is a link between concentrations of maternal exposure to air pollution to the amount of black carbon in placental cells.

Primary Endpoint

I. Evaluation of placental macrophages and the presence of black carbon.

5. METHODOLOGY

Inclusion Criteria

1. Term pregnancy (≥37 week gestation)
2. Live birth delivery by elective Caesarean section
3. Age 18-50

Exclusion Criteria

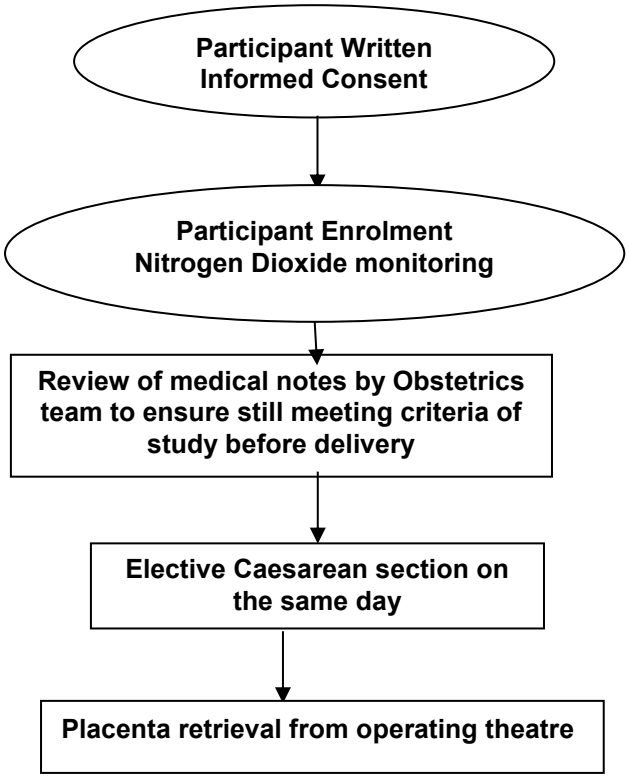
1. Complicated pregnancy
    a. Oligohydramnios
    b. Pre-eclampsia
    c. Intra-uterine growth restriction (IUGR)
2. Abnormal placental perfusion – reduced, absent or reversed end diastolic flow on Doppler study
3. Maternal request to take placenta home

Study Design / Plan

Participants (20 in total) will be expectant mothers, with an uncomplicated pregnancy, who proceed to term delivery by elective Caesarean section. They will be recruited from the maternity unit at the Royal London Hospital. Potential participants will be identified and approached in the first instance by a member of their usual clinical care team. A research physician will then review and ensure each participant meets the eligibility criteria before entering into the study. With informed consent, provided the placenta is not needed for other medical purposes, the placenta will be retrieved from the operating theatre immediately after delivery. The participant will be asked to wear a nitrogen dioxide diffusion monitor on their clothes for 2 weeks.

Duration of participation will start from recruitment antenatally to the delivery of the placenta (maximum 9 months).

Study Scheme Diagram



6. STUDY PROCEDURES

Participants (10 in total) will be expectant mothers, with an uncomplicated pregnancy, who proceed to term delivery (at least 37 weeks of pregnancy) by planned Caesarean section. Pregnant women will be recruited from the maternity unit at the Royal London Hospital.

Enrolment

Potential participants will be identified and approached by a member of their usual clinical care team. A study physician will then review and ensure each participant meets the eligibility criteria before enrolling into the study.

Informed Consent Procedures

Once participants are identified as eligible, patient information sheets will be given to them on the day of their elective Caesarean section. Informed consent will be gained from participants on the same day. At the Royal London Hospital, patients are often asked to attend the hospital at 7am on the morning of surgery and will therefore have a few hours on the ward before heading to the operating theatre. Their partners will often accompany them - this presents an opportunity to explain to both the patient and their partner about the study, allowing them to consider and ask questions before consenting.

Procedure for Collecting Data

As part of the consent form, participants will be informed that their medical records will be reviewed by their direct care team and the research doctors, in order to ensure they fit the study criteria - this will only take place after obtaining informed consent. All participants will be allocated a participant ID which will be used to label samples within the study. The only document linking their personal information and participant ID is the consent form – one copy will be given to the participant, one copy will be kept in their medical records and one copy will be kept in the investigator's site file, securely stored within the Blizard Institute, accessed only by the research team. No other information from the participants will be used.

Subject withdrawal

Should participants withdraw consent from further study involvement, we will seek their permission to include their sample results within the study. Unless they object, the data from their samples will remain on file and will be included in the final study analysis.

In line with Good Clinical Practice guidelines, at the end of the study, the results data will be securely archived for a minimum of 20 years. Arrangements for confidential destruction will then be made.

Schedule of intervention

Participants will be asked to wear a Nitrogen Dioxide Diffusion Monitor badge on their clothes for 2 weeks after informed consent. Medical notes will be reviewed by the research doctor prior to delivery to ensure all inclusion/ exclusion criteria still apply. Following delivery of the placenta, it will be washed and transferred in a specimen jar at 4^0C to the Blizard Institute for processing.

Following analysis, the findings of the study will be shared with the participants and their direct care team.

Assessment	Recruitment phase	Placenta retrieval (Delivery)
Dissemination of information leaflets	x	
Informed consent	x	
Nitrogen Dioxide Monitoring	x	
Placenta collection		x

End of Study Definition

End of study will be when data collection is complete. Participants' participation will complete before this - their participation will end when placenta is retrieved and nitrogen dioxide monitoring has completed.

It is not anticipated that there will be cause for premature termination of the study. Should any subject experience a serious adverse event (AE) associated with or attributable to any study procedure, the study status will then be reviewed.

The project will be part of the Principle Investigator (Dr Norrice Liu)'s PhD degree, under the supervision of Chief Investigator (Professor Jonathan Grigg). Principle Investigator (Dr Norrice Liu) and Co-Investigator (Professor Shakila Thangaratinam) will undertake the roles of providing information regarding study (information sheets) and obtaining informed consent. Principle Investigator (Dr Norrice Liu) will sample and process the placentas, and analyse data.

STATISTICAL CONSIDERATIONS

The main objective of this pilot study is to detect the presence of black carbon in placental macrophages. This study aims to generate data for hypothesis testing, therefore a formal sample size calculation has not been done. The aim is to generate data to inform a subsequent larger study, if black carbon is detected in placental macrophages.

In the time period of the study, recruitment of 20 pregnant women is feasible. Since this is a pilot study, the study population will not be representative of the general population. The 20 participants will be the first 20 pregnant women who are eligible for the study, who provide informed consent, during the recruitment phase.

ETHICS

No significant ethical, legal or management issues expected for this project. The procedures proposed in the project are ethically acceptable.

Ethical Review

The protocol will be reviewed both within the Sponsor Institution and by an independent research ethics committee. All the techniques are ethically acceptable.

Rationale for research

Ethical research must be informed by existing research, and investigate an important question. We have addressed this issue by thorough review of the existing literature, and by conducting appropriate background studies.

Design of research

Ethical research must employ the most appropriate design in order to answer the research question. A cross-sectional study design has been chosen for this study.

Minimisation of inconvenience, discomfort and risk for participants

Ethical research must seek to minimize potential inconvenience, discomfort and risk that participants may experience during the course of a study. The principle inconveniences of the study to the participants are minimised through use of qualified and trained practitioners to collect placenta specimens. Inconvenience is further reduced by recruiting participants during their routine hospital visits, and by performing sampling immediately after delivery.

SAFETY CONSIDERATIONS:
No additional risks or harm are anticipated on the participants.

Investigator safety
Investigators will prevent personal exposure to airborne or blood-borne infectious organisms by appropriate use of masks, goggles, lab coats and gloves while obtaining and processing samples.

DATA HANDLING AND RECORD KEEPING:
Confidentiality
Ethical research projects should ensure that participants' personal data remain confidential. Our procedures for handling, processing, storage and destruction of data are compliant with the Data Protection Act 1998. Participants will be allocated a participant ID which will be used as a code to identify them on all study samples. The only document that will link the participant's personal details with the participant ID is the consent form, which will have 3 copies – one to be given to the patient, one to be kept in their medical records, one to be kept in the investigator's site files which will be locked in a cabinet within the Blizard institute, only accessed by research team. Data will be stored in paper and electronic formats. Data stored on university computers will be de-identified. For Audit purposes, Sponsors/monitors will need to view information.

Record Retention and Archiving
Data stored in university computers will be anonymised. In line with Good Clinical Practice guidelines, at the end of the study, data will be securely archived for a minimum of 20 years. Arrangements for confidential destruction will then be made.

LABORATORIES

Blizard Institute, London

Lab Procedures
Following elective Caesarean section, the placenta and umbilical cord are usually examined for size, shape, colour, smell and structure. If no abnormalities or clinical indications identified, the placenta is usually discarded.

Participant's placenta will be examined as routine. If no other medical use/ need is identified, the placenta will be rinsed and transferred to a sterile container at 4^0C to the Blizard Institute. The specimen will be processed on the same day. Specimen will be placed in enzyme solution for digestion before further washing. Cell suspensions will then be enriched for macrophages using discontinuous density gradients. Cells will be harvested and washed before resuspending (7,8).

Cytospins stained by Diff-Quik are analysed for macrophage black carbon using digital colour images of 50 randomly selected macrophages from each placenta, captured at x100 magnification [101]. The total area of carbon in each macrophage is calculated by measuring the black content of each cell. Mean AM carbon per placenta is then calculated from the 50 macrophages.

Data Preparation and Collection
Samples will be labelled with the participant ID given to each participant. Date of collection and conditions at which the samples are sent and storied will be recorded to ensure integrity. Samples are collected from the Royal London Hospital, then stored and processed at the Blizard institute. All specimens will be discarded following analysis.

7. PRODUCTS, DEVICES, TECHNIQUES AND TOOLS

Devices

Nitrogen Dioxide Monitoring

Pollution Monitoring

A NO_2 diffusion monitor (the size of a small badge) will be placed near child for 2 weeks to monitor their NO_2 exposure. This can be placed on the buggy/ parent/ carer or on child's clothes, depending on their age and level of cooperation. An additional NO_2 badge will be placed inside the participant's home to detect indoor natural gas exposure. The data will be analysed by the manufacturers of the NO_2 diffusion monitor (IVL Swedish Environmental Research Institute Ltd, P.O. Box 53021, SE-400 14 Gothenburg, Sweden) (http://www.diffusivesampling.ivl.se/oursamplers.4.75d7780712240e747ea80004619.html)

All devices are already owned by the research team at the Blizard Institute so no purchase is required.

Tools

No tool is needed for the study.

Medicinal product

No medicinal product will be used for the study.

SAFETY REPORTING

Adverse Events (AE)

An AE is any untoward medical occurrence in a subject to whom a medicinal product has been administered, including occurrences which are not necessarily caused by or related to that product. An AE can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding), symptom or disease temporarily associated with study activities.

Notification and reporting Adverse Events or Reactions

If the AE is not defined as SERIOUS, the AE is recorded in the study file and the participant is followed up by the research team. The AE is documented in the participants' medical notes (where appropriate).

Serious Adverse Event (SAE)

In other research other than CTIMPs, a serious adverse event (SAE) is defined as an untoward occurrence that:

(a) results in death;
(b) is life-threatening;
(c) requires hospitalisation or prolongation of existing hospitalisation;
(d) results in persistent or significant disability or incapacity;
(e) consists of a congenital anomaly or birth defect; or
(f) is otherwise considered medically significant by the investigator.

An SAE occurring to a research participant should be reported to the main REC where in the opinion of the Chief Investigator the event was:

• Related – that is, it resulted from administration of any of the research procedures, and
• Unexpected – that is, the type of event is not listed in the protocol as an expected occurrence.

Notification and Reporting of Serious Adverse Events

Serious Adverse Event (SAEs) that are considered to be 'related' and 'unexpected' are to be reported to the sponsor within 24 hours of learning of the event and to the Main REC within 15 days in line with the required timeframe. For further guidance on this matter, please refer to NRES website and JRMO SOPs

Urgent Safety Measures

The CI may take urgent safety measures to ensure the safety and protection of the clinical trial subjects from any immediate hazard to their health and safety,. The measures should be taken immediately. In this instance, the approval of the REC prior to implementing these safety measures is not required. However, it is the responsibility of the CI to inform the sponsor and Main Research Ethics Committee (via telephone) of this event immediately.

The CI has an obligation to inform both the Main REC in writing within 3 days, in the form of a substantial amendment. The sponsor (Joint Research Management Office [JRMO]) must be sent a copy of the correspondence with regards to this matter. For further guidance on this matter, please refer to NRES website and JRMO SOPs.

Annual Safety Reporting

The CI will send the Annual Progress Report to the main REC using the NRES template (the anniversary date is the date on the MREC "favourable opinion" letter from the MREC) and to the sponsor. Please see NRES website and JRMO SOP for further information.

Overview of the Safety Reporting responsibilities

The CI/PI has the overall pharmacovigilance oversight responsibility. The CI/PI has a duty to ensure that safety monitoring and reporting is conducted in accordance with the sponsor's requirements.

MONITORING &AUDITING

A data monitoring committee will not be convened. Intermittent random audit of data quality will be performed by members of the investigating team under the supervision of the Chief investigator (CI).

Research sponsor will ensure arrangements and systems are in place for the management and monitoring of research.

The arrangements will be in accordance to the Research Governance Framework.

TRIAL COMMITTEES

This study is not a clinical trial – a trial committee is therefore not required. However there will be a study committee consisting of the Chief Investigator and representatives from the Research and Development Department – they will meet quarterly to ensure the study is progressing satisfactorily.

FINANCE AND FUNDING

This study is funded by Barts Charity (grant reference: MGU0312).

INDEMNITY

This study will be sponsored by Queen Mary University of London. Contact details as previously stated. **Queen Mary University of London** will arrange for suitable indemnity for negligent harm arising as a result of participation in this study to be in place.

DISSEMINATION OF RESEARCH FINDINGS:

The results will be reported and disseminated to the clinical and scientific community through ways such as peer reviewed scientific journals, internal reports or conference presentations.

Any manuscript reporting study findings will be prepared according to CONSORT guidelines and submitted to peer-reviewed biomedical journals according to ICMJE Uniform Requirements. Authorship will be based on individuals' contribution to study design, conduct, analysis, drafting/revision of manuscript and final approval of the version to be published. Authorship will not necessarily be restricted to individuals named on this protocol; neither is authorship guaranteed to any individual named on this protocol. Contributors who do not meet authorship criteria will be listed in 'Acknowledgements'.

The results will also be available to participants. They will be given contact information of the research teams.

8. REFERENCES

1. Korten I, Ramsy K, Latzin P, et al. Air Pollution during pregnancy and lung development in the child. *Paediatr Respir Rev* 2016; 10.1016/j.prrv.2016.08.008
2. Latzin P, Roosli M, Huss CE, et al. Air pollution during pregnancy and lung function in newborns: a birth cohort study. *Eur Respir J* 2009;33:594–603
3. Proietti E, Roosli M, Urs F, et al. Air Pollution During Pregnancy and Neonatal Outcome: A Review. *J Aerosol Med Pulm Drug Deliv* 2013;26(1):9-23
4. Pedersen M, Giorgis-Allemand L, Bernard C, et al. Ambient air pollution and low birthweight: a European cohort study (ESCAPE). *Lancet Respir Med* 2013;1(9):695–704
5. Wick P, Malek A, Manser P, et al. Barrier capacity of human placenta for nanosized materials. *Environ Health Perspect 2010;*118:432-436
6. Pinhal-Enfield G, Vasan NS, Leibovich SJ, et al. The Role of Macrophages in the Placenta. *Embryology - Updates and Highlights on Classic Topics* 2012;Chapter 6
7. Sutton LN, Mason, DY, Redman CW, et al. Isolation and characterisation of human fetal macrophages from placenta. *Clin Exp Immunol* 1989;78(3):437-443
8. Jurado KA, Simoni MK, Tang Z, et al. Zika virus productively infects primary human placenta-specific macrophages. JCI Insight 2016 Aug18:1(13)
9. Kulkarni N, Pierse N, Rushton L, et al. Carbon in airway macrophages and lung function in children. *N Engl J Med* 2006;355:21-30

9. APPENDIX

Nitrogen Dioxide Monitoring:
http://www.diffusivesampling.ivl.se/oursamplers.4.75d7780712240e747ea80004619.html

Appendix 9 – APPIP study: Participant information sheet

PATIENT INFORMATION SHEET Version 1.3 28/07/2017
REC reference: 17/NW/0092
IRAS number: 219053
Name of researcher: Dr Norrice Liu

PART 1

1. **Invitation**
   You are being invited to take part in a pilot research study. Before you decide whether or not to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully, and discuss it with others if you wish.

   PART 1 tells you the purpose of this study and what will happen if you take part.

   PART 2 gives you more detailed information about the conduct of the study.

   Please ask us if there is anything that is not clear, or if you would like more information. Take time to decide whether or not you wish to take part.

2. **What is the purpose of the study?**
   Air pollution, such as emissions from petrol and diesel car engines, is linked to a wide range of adverse health effects. Exposure to air pollution during pregnancy can affect the unborn baby. The baby's breathing system starts to develop from about 4 weeks of pregnancy, this continues until their early adulthood, and can be affected by air pollution. There is a link between mothers who are exposed to high level of air pollution during pregnancy and outcomes such as small babies (lower birth weight) and breathing problems later in the baby's life. It is uncertain whether the pollution particles can be passed directly across the placenta from the mother to the baby.

3. **Why have I been chosen?**
   You have been chosen because you have a relatively uncomplicated pregnancy, and you live in or around London - London is known to be a well polluted area.

4. **Do I have to take part?**
   No. It is up to you to decide whether or not to take part. If you decide that you would like to take part, then you will be given this information sheet to keep and be asked to sign a consent form to confirm that you understand what is involved when taking part in this study. If you decide to take part you are free to leave the study at any time and without giving a reason. If you withdraw, unless you object, we will still keep records relating to your participation up to that point, as this is valuable to the study. A decision to withdraw at any time, or a decision not to take part, will not affect the quality of any care you may require from us in the future.

5. **What will happen to me if I take part?**
   If you are interested in taking part, trained researchers will arrange a time to meet you at the Royal London Hospital – this can coincide with your next hospital visit if you have one pending. With your consent, the research doctor will review your antenatal record to ensure

you fit the enrolment criteria of the study. You will receive your routine antenatal care as planned. This study will not add extra procedures/ appointments.

You will be asked to wear a small **pollution monitor badge** for 2 weeks – this can be attached to your clothes to detect your pollution exposure level. The badge will be collected by the researcher at the end of the 2 weeks.

Pollution Monitoring Badge

Usually following delivery of the baby, the placenta will be delivered. The placenta is usually looked at by the midwife before it is discarded. If the placenta looks normal and no further medical intervention is needed, the research team will take the placenta to the research institute (Blizard Institute) for processing and analysis. The study report will be fed back to you after the results are analysed.

6. **What do I have to do?**
   If you agree to take part, you can sign a consent form and give it back to the Obstetrics team or the research doctor. We will then collect the placenta after your delivery. There is nothing extra you will need to do. Your participation will be from recruitment til the collection of the placenta and completion of the 2 week pollution monitoring (maximum 9 months).

7. **What are the alternatives for testing?**
   Extracting cells directly from the placenta is the only way to detect the presence of air pollution particles.

8. **What are other possible disadvantages and risks of taking part?**
   There is no extra treatment or procedure other than your routine antenatal care, so no additional disadvantages or side effects are anticipated.

9. **What are the possible benefits of taking part?**
   This study will help us understand whether air pollution particles that mothers are exposed to on a daily basis, are passed directly to the baby, as it will provide no information of clinical significance for you and your baby. The pollution monitor badge will provide you with information regarding your personal exposure to pollution over the course of 2 weeks.

   Barts Health NHS Trust (The Royal London Hospital) has been working with Global Action Plan to reduce patients' personal exposure to air pollution. We think this study will influence in trying to reduce air pollution exposure and limit its effect on expectant mothers and their babies.

10. **What happens when the research study stops?**
    The placenta will be disposed of following analysis – the study report will be published in a medical journal.

11. **What if there is a problem?**
    If you have concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your question. Our contact details can be found at the bottom of this sheet. If you remain unhappy and wish to complain formally, you can do this through either the NHS Complaints Procedure (details are at the end of this sheet).

    Queen Mary University of London has agreed that if you are harmed as a result of your participation in the study, you will be compensated, provided that, on the balance of probabilities, an injury was caused as a direct result of the intervention or procedures you

received during the course of the study. These special compensation arrangements apply where an injury is caused to you that would not have occurred if you were not in the study. These arrangements do not affect your right to pursue a claim through legal action.

12. **Will my taking part in this study be kept confidential?**
    Yes. All the information about your participation in this study will be kept confidential. (See Part 2)

This completes Part 1 of the Information Sheet. If the information has interested you and you would like to participate, please continue to read the additional information in Part 2 before making any decision.

PART 2

13. **What if new information becomes available?**
    Sometimes during the course of a study, new information becomes available on the procedures that are being studied. If this happens, we will tell you about it and discuss with you whether you want to or should continue in the study. If you decide to withdraw, you will suffer no adverse effects as a result. If you decide to continue in the study you may be asked to sign an updated consent form.

    On receiving new information, we might consider it to be in your best interests to withdraw you from the study. If so, we will explain the reasons and arrange for your care to continue. If the study is stopped for any other reason, you will be told why.

14. **Will my part in this study be kept confidential?**
    You will be allocated a participant ID, which will be used to label the placenta. Data will be stored in paper and electronic formats, data generated will be analysed by the research team - all de-identified and securely stored within the Blizard Institute, only accessed by the research team. All information will be held securely at the research centre and the Royal London Hospital under the provisions of the 1998 Data Protection Act.

    Your data records will be available to people authorised to work on the study but may also need to be made available to people authorised by the Research Sponsor, which is the organisation responsible for ensuring that the study is carried out correctly. The information collected about you may also be shown to authorised people from the UK Regulatory Authority and Independent Ethics Committee; this is to ensure that the study is carried out to the highest possible scientific standards. All will have a duty of confidentiality to you as a research participant.

    By signing the consent form you agree to this access for the current study and any further research that may be conducted in relation to it, even if you withdraw from the current study.

    If you withdraw consent from further study involvement, we will seek your permission to include your sample results within the study. We will not do so without your permission. Unless you object, your data and samples will remain on file and will be included in the final study analysis.

    In line with Good Clinical Practice guidelines, at the end of the study, your data will be securely archived for a minimum of 20 years. Arrangements for confidential destruction will then be made.

15. **What will happen to the sample I give?**
The placenta will be labelled with the participant ID, and transported to the Blizard Institute for processing and analysis. We will extract cells called macrophages, which are usually involve in removing unwanted particles or substances, from the placenta and look for the presence of air pollution particles.

The pollution monitoring badges will be analysed in Sweden (IVL Swedish Environmental Research Institute Ltd, P.O. Box 53021, SE-400 14 Gothenburg, Sweden). No samples will be sent to this location. The devices will only have anonymised data prior to transport.

16. **What will happen to the results of this study?**
The results of the study will be available after it finishes and will usually be published in a medical journal or be presented at a scientific conference. The data will be anonymous, you will not be identified in any report or publication. Should you wish to see the results, please ask your study doctor.

17. **Who is organising and funding this study?**
The study is sponsored by Queen Mary University of London, and it is funded by Barts Charity (https://bartscharity.org.uk/).

18. **Who has reviewed the study?**
Before any research goes ahead it has to be checked by an independent Research Ethics Committee. They make sure that the research is fair. Your project has been checked by the London - Bloomsbury Research Ethics Committee. (REC reference: 17/NW/0092)

19. **Contact for further information**
You are encouraged to ask any questions you wish, before, during or after your participation. If you have any questions about the study, please speak to the research team, who will be able to provide you with up to date information about the study. If you wish to read the research on which this study is based, please ask your research team. If you require any further information or have any concerns while taking part in the study please contact the research team (contact details are at the end of this sheet).

If you decide you would like to take part then please read and sign the consent form, which will be the only document linking your name to the participant ID. You will be given a copy of this information sheet and the consent form to keep. One copy of the consent form will be given to you, one copy to be stored in medical records and one copy to be kept within the investigator's site file.

Thank you for taking the time to read this information sheet and to consider this study.

Research Team:

Chief Investigator:
Professor Jonathan Grigg, 07787 550775, j.grigg@qmul.ac.uk
Clinical Research Fellow:
Dr Norrice Liu, 020 7882 2616, n.liu@qmul.ac.uk

For advice about taking part in research in the NHS:
INVOLVE
Wessex House
Upper Market Street
Eastleigh, Hampshire
SO50 9FD
Telephone: 023 8065 1088
Textphone: 023 8062 6239
Fax: 023 8065 2885
Email: admin@invo.org.uk

For advice about research and patient issues at The Royal London Hospital and Barts Health NHS Trust:
Patient Advice and Liaison Service (PALS):
Ground Floor, Front Block
The Royal London Hospital
Whitechapel Road, London E1 1BB
Tel: 020 7943 1335
Fax: 020 7377 7361
Minicom: 020 7943 1350
E-mail: PALS@bartshealth.nhs.uk

Appendix 10 – APPIP: Consent form

AirPollutionParticlesinPlacenta

RECreference:17/NW/0092 **IRASnumber: 219053**
Nameofresearcher:DrNorriceLiu

ParticipantID:_____

CONSENTFORM

	ParticipantconsentformVersion1.3 28/07/2017	**Please initial allboxes**
1	I confirm that I have read and understood the information sheet dated 28/07/2017version1.3 fortheabovestudy.Ihavehadtheopportunityto consider the information, ask questions and have had these answered satisfactorily.	
2	I understan d that my participation is voluntary and that I am free to withdrawatanytime,withoutgivingareason,andwithoutmycareorlegal rightsbeingaffected.	
3	Iagreetocarryapollutionmonitortoevaluatemypersonalexposureto air pollution. I und erstand the monitor badge will be analysed in a laboratoryinSwedenbut thedevicewillonlyhaveanonymiseddataprior totransport.	
4	Iunderstandthatrelevantsectionsofmymedicalnotesanddatacollected duringthestudymaybelookedatbyresea rchdoctorsfromQueenMary University London, from regulatory authorities or from the NHS Tru st, whereitisrelevantformy takingpartinthisresearch.Igivepermissionfor thesedoctorstohaveaccess to myrecords.	
5	Iagreefortheplacentaofm ycurrentpregnancytobecollectedfollowing delivery. Iunderstandtheplacentawillbedisposedofafteranalysis.	
6	I agree to take part in this study. I understand I will be assigned a participantIDwhichwillbeused toidentifymewithinthestu dy,andIwill notbeidentifiedinanyreportorpublicationofthestudyresults.	

NameofParticipant	Signature	Date
NameofResearcher	Signature	Date

Appendix 11 – Ethics approval and correspondence

North West - Greater Manchester West Research Ethics Committee
Barlow House
3rd Floor
4 Minshull Street
Manchester
M1 3DZ

NHS Health Research Authority

Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

09 February 2017

Professor Jonathan Grigg
Blizard Institute, Queen Mary University of London
4 Newark St
London
E1 2AT

Dear Professor Grigg

Study title:	**Presence of Air Pollution Particles in Placental Macrophages**
REC reference:	**17/NW/0092**
IRAS project ID:	**219053**

The Proportionate Review Sub-committee of the North West - Greater Manchester West Research Ethics Committee reviewed the above application on 03 February 2017.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact hra.studyregistration@nhs.net outlining the reasons for your request. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Ethical opinion

On behalf of the Committee, the sub-committee gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

The sub-committee would like the second sentence in point 3 of the PIS, '- London is known to have some of the most polluted air in Western Europe.', to be changed to 'London is known to be a well polluted area'.
The sub-committee would like the last sentence on paragraph 6 of the PIS to be removed, 'This study aims to look into whether mother's exposure to air pollution will result in the presence of pollution particles within the placenta, thereby having possible direct effect on birth outcomes'.
The sub-committee would like point 9 of the PIS to include the following after the first sentence, 'As it will provide no information of clinical significance for you and your baby'.
The sub-committee would like the word 'findings' in point 5 and point 10 of the PIS to be replaced with 'study report'.

You should notify the REC once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. Revised documents should be submitted to the REC electronically from IRAS. The REC will acknowledge receipt and provide a final list of the approved documentation for the study, which you can make available to host organisations to facilitate their permission for the study. Failure to provide the final versions to the REC may cause delay in obtaining permissions.

Management permission must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for HRA Approval (England)/ NHS permission for research is available in the Integrated Research Application System, www.hra.nhs.uk or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database. This should be before the first participant is recruited but no later than 6 weeks after recruitment of the first participant.

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from the HRA. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see
"Conditions of the favourable opinion").

Summary of discussion at the meeting

Ethical issues raised, noted and resolved in discussion:

Informed consent process and the adequacy and completeness of participant information

The sub-committee were of the opinion the PIS well written and raised no ethical issues. The sub-committee had noted a few minor changes they would like to be made. Please see these changes mentioned below in the decision.

Approved documents

The documents reviewed and approved were:

Document	*Version*	*Date*
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Verification of Insurance]		15 July 2016
IRAS Application Form [IRAS_Form_19012017]		19 January 2017
IRAS Application Form XML file [IRAS_Form_19012017]		19 January 2017
IRAS Checklist XML [Checklist_19012017]		19 January 2017
Letter from funder [Barts Charity letter]		20 December 2016

Letter from sponsor [Sponsorship agreement]		19 January 2017
Other [Case Report Form]	1.0	12 December 2016
Participant consent form [Consent]	1.1	12 January 2017
Participant information sheet (PIS) [PIS]	1.1	12 January 2017
Research protocol or project proposal [Air Pollution Particles in Placenta Protocol]	1.2	13 January 2017
Summary CV for Chief Investigator (CI) [CV J.Grigg]	1.0	08 November 2016
Summary CV for student [CV N.Liu]	1.0	08 November 2016
Summary CV for supervisor (student research) [CV J.Grigg]	1.0	08 November 2016

Membership of the Proportionate Review Sub-Committee

The members of the Sub-Committee who took part in the review are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

Notifying substantial amendments
Adding new sites and investigators
Notification of serious breaches of the protocol
Progress and safety reports
Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

17/NW/0092	**Please quote this number on all correspondence**

Yours sincerely

Dr Lorraine Lighton (Chair) Chair

Email: nrescommittee.northwest-gmwest@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

"After ethical review – guidance for researchers" [SL-AR2]

Copy to: *Dr Sally Burtles, Elizabeth Clough, Barts Health NHS Trust*

North West - Greater Manchester West Research Ethics Committee

Attendance at PRS Sub-Committee of the REC meeting on 03 February 2017

Committee Members:

Name	*Profession*	*Present*	*Notes*
Mrs Seonaid Beddows	Research Governance and Administration Manager	Yes	
Dr Lorraine Lighton (Chair)	Retired Consultant in Communicable Disease Control	Yes	
Dr Gideon Smith	Consultant in Public Health Medicine	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Miss Anna Bannister	REC Manager
Miss Nafeesa Khanam	REC Assistant

North West - Greater Manchester West Research Ethics Committee
Barlow House
3rd Floor
4 Minshull Street
Manchester
M1 3DZ

Health Research Authority

Please note: This is an acknowledgement letter from the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

15 February 2017

Professor Jonathan Grigg
Blizard Institute, Queen Mary University of London
4 Newark St
London
E1 2AT

Dear Professor Grigg

Study title:	**Presence of Air Pollution Particles in Placental Macrophages**
REC reference:	**17/NW/0092**
IRAS project ID:	**219053**

Thank you for your letter of 10/02/2017. I can confirm the REC has received the documents listed below and that these comply with the approval conditions detailed in our letter dated 09 February 2017

Documents received

The documents received were as follows:

Document	*Version*	*Date*
Covering letter on headed paper [Cover letter]	1.0	10 February 2017
IRAS Checklist XML [Checklist_10022017]		10 February 2017
Participant consent form [Consent]	1.2	10 February 2017
Participant information sheet (PIS) [PIS]	1.2	10 February 2017

Approved documents

The final list of approved documentation for the study is therefore as follows:

Document	*Version*	*Date*

Covering letter on headed paper [Cover letter]	1.0	10 February 2017
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Verification of Insurance]		15 July 2016
IRAS Application Form [IRAS_Form_19012017]		19 January 2017
IRAS Checklist XML [Checklist_19012017]		19 January 2017
IRAS Checklist XML [Checklist_10022017]		10 February 2017
Letter from funder [Barts Charity letter]		20 December 2016
Letter from sponsor [Sponsorship agreement]		19 January 2017
Other [Case Report Form]	1.0	12 December 2016
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Summary CV for student [CV N.Liu]	1.0	08 November 2016
Summary CV for supervisor (student research) [CV J.Grigg]	1.0	08 November 2016

You should ensure that the sponsor has a copy of the final documentation for the study. It is the sponsor's responsibility to ensure that the documentation is made available to R&D offices at all participating sites.

17/NW/0092 Please quote this number on all correspondence

Yours sincerely

Nafeesa Khanam REC Assistant

E-mail: nrescommittee.northwest-gmwest@nhs.net

Copy to: Dr Sally Burtles
Elizabeth Clough, Barts Health NHS Trust

NHS
Health Research Authority

Professor Jonathan Grigg
Blizard Institute, Queen Mary University of London
4 Newark St
London
E1 2AT

Email: hra.approval@nhs.net

20 March 2017
Amended and Reissued 23 March 2017

Dear Professor Grigg,

Letter of HRA Approval

Study title:	**Presence of Air Pollution Particles in Placental Macrophages**
IRAS project ID:	**219053**
REC reference:	**17/NW/0092**
Sponsor	**Queen Mary University of London**

I am pleased to confirm that **HRA Approval** has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications noted in this letter.

Participation of NHS Organisations in England
The sponsor should now provide a copy of this letter to all participating NHS organisations in England.

Appendix B provides important information for sponsors and participating NHS organisations in England for arranging and confirming capacity and capability. **Please read *Appendix B* carefully**, in particular the following sections:
Participating NHS organisations in England – this clarifies the types of participating organisations in the study and whether or not all organisations will be undertaking the same activities
Confirmation of capacity and capability - this confirms whether or not each type of participating NHS organisation in England is expected to give formal confirmation of capacity and capability. Where formal confirmation is not expected, the section also provides details on the time limit given to participating organisations to opt out of the study, or request additional time, before their participation is assumed.
Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria) - this provides detail on the form of agreement to be used in the study to confirm capacity and capability, where applicable.
Further information on funding, HR processes, and compliance with HRA criteria and standards is also provided.

It is critical that you involve both the research management function (e.g. R&D office) supporting each organisation and the local research team (where there is one) in setting up your study. Contact details

and further information about working with the research management function for each organisation can be accessed from www.hra.nhs.uk/hra-approval.

Appendices
The HRA Approval letter contains the following appendices:
A – List of documents reviewed during HRA assessment
B – Summary of HRA assessment

After HRA Approval
The document *"After Ethical Review – guidance for sponsors and investigators",* issued with your REC favourable opinion, gives detailed guidance on reporting expectations for studies, including:
Registration of research
Notifying amendments
Notifying the end of the study
The HRA website also provides guidance on these topics, and is updated in the light of changes in reporting expectations or procedures.

In addition to the guidance in the above, please note the following:
HRA Approval applies for the duration of your REC favourable opinion, unless otherwise notified in writing by the HRA.
Substantial amendments should be submitted directly to the Research Ethics Committee, as detailed in the *After Ethical Review* document. Non-substantial amendments should be submitted for review by the HRA using the form provided on the HRA website, and emailed to hra.amendments@nhs.net.
The HRA will categorise amendments (substantial and non-substantial) and issue confirmation of continued HRA Approval. Further details can be found on the HRA website.

Scope
HRA Approval provides an approval for research involving patients or staff in NHS organisations in England.

If your study involves NHS organisations in other countries in the UK, please contact the relevant national coordinating functions for support and advice. Further information can be found at http://www.hra.nhs.uk/resources/applying-for-reviews/nhs-hsc-rd-review/.

If there are participating non-NHS organisations, local agreement should be obtained in accordance with the procedures of the local participating non-NHS organisation.

User Feedback
The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/.

HRA Training
We are pleased to welcome researchers and research management staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

Your IRAS project ID is **219053**. Please quote this on all correspondence.

Yours sincerely

Thomas Fairman
HRA Assessor

Email: hra.approval@nhs.net

Copy to: Dr Sally Burtles, Queen Mary University of London, (Sponsor Contact)
Ms Elizabeth Clough, R&D Governance Operations Manager, Barts Health, (Lead NHS R&D Contact)

Appendix A - List of Documents

The final document set assessed and approved by HRA Approval is listed below.

Document	*Version*	*Date*
Covering letter on headed paper [Cover letter]		10 February 2017
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Verification of Insurance]		15 July 2016
IRAS Application Form [IRAS_Form_19012017]		19 January 2017
Letter from funder [Barts Charity letter]		20 December 2016
Letter from sponsor [Sponsorship agreement]		19 January 2017
Other [Case Report Form]	1.0	12 December 2016
Other [Response to HRA request for clarifications]		20 March 2017
Other [Sponsor confirmation of non-substantial amendment]		20 March 2017
Participant consent form [Consent]	1.2	10 February 2017
Participant information sheet (PIS) [PIS]	1.2	10 February 2017
Research protocol or project proposal [Air Pollution Particles in Placenta Protocol]	1.2	13 January 2017
Summary CV for Chief Investigator (CI) [CV J.Grigg]		08 November 2016
Summary CV for student [CV N.Liu]		08 November 2016
Summary CV for supervisor (student research) [CV J.Grigg]		08 November 2016

Appendix B - Summary of HRA Assessment

This appendix provides assurance to you, the sponsor and the NHS in England that the study, as reviewed for HRA Approval, is compliant with relevant standards. It also provides information and clarification, where appropriate, to participating NHS organisations in England to assist in assessing and arranging capacity and capability.
For information on how the sponsor should be working with participating NHS organisations in
England, please refer to the, *participating NHS organisations, capacity and capability* and *Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria)* sections in this appendix.
The following person is the sponsor contact for the purpose of addressing participating organisation questions relating to the study:

Name: Dr Sally Burtles
Email: sponsorsrep@bartshealth.nhs.uk

HRA assessment criteria

Section	HRA Assessment Criteria	Compliant with Standards	Comments
1.1	IRAS application completed correctly	Yes	No comments
2.1	Participant information/consent documents and consent process	Yes	No comments
3.1	Protocol assessment	Yes	No comments
4.1	Allocation of responsibilities and rights are agreed and documented	Yes	This is a non-commercial single site study taking place in the NHS where that single NHS organisation's partner University is the study sponsor. Therefore no study agreements are expected.
4.2	Insurance/indemnity arrangements assessed	Yes	Where applicable, independent contractors (e.g. General Practitioners) should ensure that the professional indemnity provided by their medical defence organisation covers the activities expected of them for this research study
Section	**HRA Assessment Criteria**	**Compliant with Standards**	**Comments**
4.3	Financial arrangements assessed	Yes	External study funding has been secured from Barts Charity.
5.1	Compliance with the Data Protection Act and data security issues assessed	Yes	No comments
5.2	CTIMPS – Arrangements for compliance with the Clinical Trials Regulations assessed	Not Applicable	No comments
5.3	Compliance with any applicable laws or regulations	Yes	No comments

6.1	NHS Research Ethics Committee favourable opinion received for applicable studies	Yes	REC Favourable Opinion was issued by the Greater Manchester West Research Ethics Committee on the 15th February 2017. Clarifications were provided by the researchers to comply with HRA Approval standards. These were classified by the sponsor as a nonsubstantial amendment.
6.2	CTIMPS – Clinical Trials Authorisation (CTA) letter received	Not Applicable	No comments
6.3	Devices – MHRA notice of no objection received	Not Applicable	No comments
6.4	Other regulatory approvals and authorisations received	Not Applicable	No comments

Participating NHS Organisations in England

This provides detail on the types of participating NHS organisations in the study and a statement as to whether the activities at all organisations are the same or different.
This is a non-commercial single site study taking place in the NHS where that single NHS organisation's partner University is the study sponsor. Therefore there is only one site type involved in the research.

If this study is subsequently extended to other NHS organisation(s) in England, an amendment should be submitted to the HRA, with a Statement of Activities and Schedule of Events for the newly participating NHS organisation(s) in England.

The Chief Investigator or sponsor should share relevant study documents with participating NHS organisations in England in order to put arrangements in place to deliver the study. The documents should be sent to both the local study team, where applicable, and the office providing the research management function at the participating organisation. For NIHR CRN Portfolio studies, the Local LCRN contact should also be copied into this correspondence. For further guidance on working with participating NHS organisations please see the HRA website.

If chief investigators, sponsors or principal investigators are asked to complete site level forms for participating NHS organisations in England which are not provided in IRAS or on the HRA website, the chief investigator, sponsor or principal investigator should notify the HRA immediately at hra.approval@nhs.net. The HRA will work with these organisations to achieve a consistent approach to information provision.

Confirmation of Capacity and Capability

This describes whether formal confirmation of capacity and capability is expected from participating NHS organisations in England.

This is a non-commercial single site study taking place in the NHS where that single NHS organisation's partner University is the study sponsor. The participating NHS organisation will therefore **be expected to formally confirm their capacity and capability to host this research according to local requirements.**
Following issue of this letter, participating NHS organisations in England may now confirm to the sponsor their capacity and capability to host this research, when ready to do so. How capacity and capacity will be confirmed is detailed in the *Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria)* section of this appendix.
The Assessing, Arranging, and Confirming document on the HRA website provides further information for sponsors and NHS organisations on assessing, arranging and confirming capacity and capability.

Principal Investigator Suitability

This confirms whether the sponsor position on whether a PI, LC or neither should be in place is correct for each type of participating NHS organisation in England and the minimum expectations for education, training and experience that PIs should meet (where applicable).

A Principal Investigator should be appointed at study sites.

GCP training is not a generic training expectation, in line with the HRA statement on training expectations.

HR Good Practice Resource Pack Expectations

This confirms the HR Good Practice Resource Pack expectations for the study and the pre-engagement checks that should and should not be undertaken

As a non-commercial single site study taking place in the NHS where that single NHS organisation's partner University is the study sponsor, it is unlikely that letters of access or honorary research contracts will be applicable, except where local network staff employed by another Trust (or University) are involved (and then it is likely that arrangements are already in place).

If members of an external research team will be attending NHS sites to conduct the study activities detailed at IRAS A18 and A19 they should obtain a Letter of Access. This would be on the basis of a Research Passport or an NHS to NHS confirmation of pre-engagement checks letter (if NHS employed). Pre-engagement checks should confirm standard DBS checks, appropriate barred list checks, and occupational health clearance.

Other Information to Aid Study Set-up

This details any other information that may be helpful to sponsors and participating NHS organisations in England to aid study set-up.

The applicant has indicated that they do not intend to apply for inclusion on the NIHR CRN Portfolio.

North West - Greater Manchester West Research Ethics Committee
Barlow House
3rd Floor
4 Minshull Street
Manchester
M1 3DZ

Tel: 0207 104 8021
22 August 2017

Dr Sally Burtles
Joint Research Management Office
Queen Mary Innovation Centre
5, Walden Street
E1 2EF

Dear Dr Burtles

Study title:	**Presence of Air Pollution Particles in Placental Macrophages**
REC reference:	**17/NW/0092**
Amendment number:	**1**
Amendment date:	**28 July 2017**
IRAS project ID:	**219053**

The above amendment was reviewed the Sub-Committee in correspondence.

Ethical opinion

The Sub-Committee reviewed the amendment and requested further information below:

How many monitors will there be, where will they be placed, and will they be used before or after the birth.
Have any mothers been recruited yet? If so, will they be re-consented to use the monitor(s).

Researcher Dr Norrice Liu explained there would be one monitor per mother, the monitor is the size of a 10p coin and can be clipped onto clothing- we would advise mothers to clip the monitor onto their outer-wear wherever they go, for the duration of two weeks. The monitors would be used around delivery time- ideally from recruitment antenatally, until the two week period has completed. Mothers are often recruited from antenatal clinic 1-2 weeks before planned
Caesarian sections so the monitor can either be collected at delivery or after delivery, depending on when the 2 week period completes. So the monitoring period would span across before/after birth but would vary with different participants. We would arrange for monitor collection in order to limit inconvenience.

He further explained that 7 participants have been recruited already and we would not reconsent them for monitor use. We would only start adding monitor usage for all new participants after new ethics approval has been granted.

The Sub-Committee were satisfied with the clarification but requested the protocol was updated to include the above wording.

Researcher Dr Norrice Liu submitted an amended protocol which the Sub-Committee reviewed and were happy they had addressed their concerns. The Sub-Committee had no further issues.

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Covering letter on headed paper		28 July 2017
Notice of Substantial Amendment (non-CTIMP)	1	28 July 2017
Participant consent form [APPIP Clean]	1.3	28 July 2017
Participant consent form [APPIP Tracking]	1.3	28 July 2017
Participant information sheet (PIS) [APPIP Clean]	1.3	28 July 2017
Participant information sheet (PIS) [APPIP Tracked]	1.3	28 July 2017
Referee's report or other scientific critique report [Case Report Form Clean]	1.1	28 July 2017
Referee's report or other scientific critique report [Case Report Form Tracked]	1.1	28 July 2017
Research protocol or project proposal [Clean]	1.4	21 August 2017
Research protocol or project proposal [Tracked]	1.4	21 August 2017

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

Working with NHS Care Organisations

Sponsors should ensure that they notify the R&D office for the relevant NHS care organisation of this amendment in line with the terms detailed in the categorisation email issued by the lead nation for the study.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our Research Ethics Committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

17/NW/0092:	**Please quote this number on all correspondence**

Yours sincerely

Dr Lorraine Lighton (Chair) Chair

E-mail: nrescommittee.northwest-gmwest@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Elizabeth Clough, R&D Governance Operations Manager Professor Jonathan Grigg*

North West - Greater Manchester West Research Ethics Committee

Attendance at Sub-Committee of the REC meeting on 14 August 2017

Committee Members:

Name	*Profession*	*Present*	*Notes*
Dr Lorraine Lighton (Chair)	Retired Consultant in Communicable Disease Control	Yes	
Dr Peter Owen	Retired Mathematics Lecturer	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Miss Anna Bannister	REC Manager

NHS
Health Research Authority

North West - Greater Manchester West Research Ethics Committee

Barlow House
3rd Floor
4 Minshull Street
Manchester
M1 3DZ

Tel: 0207 104 8021

01 June 2018

Dr Norrice Liu
Clinical Research Fellow
Blizard Institute
4 Newark St
London
E1 2AT

Dear Dr Liu

Study title:	**Presence of Air Pollution Particles in Placental Macrophages**
REC reference:	**17/NW/0092**
Amendment number:	**2**
Amendment date:	**27 April 2018**
IRAS project ID:	**219053**

- This amendment consists of changes to the consent procedure, as well as updates to the protocol and sponsor details.

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Covering letter on headed paper		04 May 2018
Notice of Substantial Amendment (non-CTIMP)	2	27 April 2018
Research protocol or project proposal [Clean]	1.4	20 April 2018
Research protocol or project proposal [Tracked]	1.4	20 April 2018

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

Working with NHS Care Organisations

Sponsors should ensure that they notify the R&D office for the relevant NHS care organisation of this amendment in line with the terms detailed in the categorisation email issued by the lead nation for the study.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our Research Ethics Committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

17/NW/0092:	**Please quote this number on all correspondence**

Yours sincerely

Dr Lorraine Lighton Chair

E-mail: nrescommittee.northwest-gmwest@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Elizabeth Clough, R&D Governance Operations Manager*
Dr Norrice Liu, Blizard Institute

North West - Greater Manchester West Research Ethics Committee
Attendance at Sub-Committee of the REC
Committee Members:

Name	*Profession*	*Present*	*Notes*
Dr Lorraine Lighton (Chair)	Retired Consultant in Communicable Disease Control	Yes	
Dr Gideon Smith	Consultant in Public Health Medicine	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Mr Ewan Waters	REC Assistant

Appendix 12 – Aethalometer activity diary

Participant's Activity Diary

To be filled in by participant / parent during carbon monitoring

Date	Time	Activity (e.g. travel, school, playtime, activities, bedtime)	Place (e.g. home, school, playground)	Mode of transport (e.g. walking, bus, tube, car)